THE PATHOBIOLOGY AND IMMUNOLOGY OF CRYPTOSPORIDIOSIS

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There were three aims of this study: firstly, to investigate cryptosporidiosis in a symptomatic laboratory animal model where diarrhoea was the most important clinical sign; secondly, to compare the infection in this model with that in lambs; thirdly, to determine the neutralizing ability of specific immunoglobulins.

Strains of rats and mice were each infected with three isolates of Cryptosporidium parvum. Using histopathological scoring methods it was shown that the distribution of infection depended more on the host species than on the isolates used. The inbred Lister rat strain was selected as a suitable laboratory animal host because infection was symptomatic with a distribution and age susceptibility which paralleled that in lambs

The infection in mice was asymptomatic and in the small intestine occurred principally in the ileum. Excystation conditions were shown to be more favourable in the mouse ileum by comparing the number of sporozoites collected from segments of intestine after oral inoculation of oocysts. In vitro experiments showed that exposure of oocysts to a p shift from approximately 3.6 to 8.4 was an important excystation stimulus. The parasite's asexual reproductive capacity was examined by counting merozoites in mouse ileal surface mucus. It was shown that the amount of surface mucus increased during infection in association with increasing numbers of merozoites. A method was described which enabled merozoites to be collected for use as antigen.

The kinetics and specificity of antibodies produced in response to Cryptosporidium infection were examined in rats and lambs using IFA and immunoblotting techniques respectively. For rats and lambs specific serum antibodies were detected within one week of oocyst inoculation and titres did not decrease over an observation period of approximately 40 days. Higher serum titres were found in rats infected at 15 days compared to those infected at 4 days of age. This may have reflected the postnatal development of the gut associated lymphoid tissue in rats whereby the younger animals were immunologically immature at the time of infection.

Numerous oocyst antigens, with molecular weights greater that 45Kd., were recognized by antibody in convalescent rat and lamb sera. In addition, two antigens with estimated molecular weights of 23Kd. and between 12.3 and 17.3Kd. were features on serum immunoblots. It was shown in lambs that declining oocyst output was associated with increasing titres of IgA and IgM in faecal extracts and that both antibodies had similar oocyst antigen specificity. Most of the merozoite antigens recognized by IgA appeared in a molecular weight range from 66 to 180Kd., as did most of the oocyst antigens recognized by this antibody. However, a 23Kd. oocyst antigen and others between 45 and 66Kd. recognized by IgA were not detected by this antibody on merozoite blots. These antigenic differences may be helpful in determining which stage of the parasite is most important to the development of protective immunity.

Mucus extracts from infected and uninfected lambs were fractionated on Bio-Gel A-1.5m. In vitro sporozoite agglutination was associated not only with fractions containing specific IgA but also glycoprotein fractions devoid of detectable specific antibody. Pretreatment of sporozoites with IgG, separated from hyperimmune lamb serum by affinity chromatography, was shown to neutralize their capacity to infect 5-day old rats. This may have important implications for passive protection of young ruminants.

To my parents for having taken chances on me and to my wife, Anne, for taking chances with me. Many thanks.

DECLARATION

I declare that this thesis has been composed entirely by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

Signed,

(Bruce D. Hill)

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CONTENTS

ABBREVIATIONS

AIDS.....acquired immunodeficiency syndrome

CAM.....chorioallantoic membrane

CM.....cell-mediated

DAB.....3,3' diaminobenzidene

ETEC.....enterotoxigenic *Escherichia coli*

FITC.....fluorescein isothiocyanate

HBSS.....Hank's balanced salt solution

HFL.....human foetal lung

HIV.....human immunodeficiency virus

HRPO.....horseradish peroxidase

Ia^+.....cells positive for the class II Ia major histocompatability complex antigen

IFA.....indirect immunofluorescence assay

Ig.....immunoglobulin

Kd.....kilo-dalton

PBS.....phosphate buffered saline

SD.....standard deviation of the mean

SDS.....sodium dodecyl sulphate

SDS-PAGE.....sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM.....standard error of the mean

shells.....*Cryptosporidium* oocyst walls after excystation

$_sIg^+$.....surface immunoglobulin positive

Tris.....2-amino-2-(hydroxymethyl)propane 1,3-diol

INTRODUCTION

Cryptosporidium is a small apicomplexan protozoan inhabiting the respiratory and gastrointestinal tracts of a wide range of vertebrates. Infection of the respiratory tract is more common in birds while gastrointestinal infection is more important in mammals.

Acute infectious diarrhoea remains an important medical and veterinary health problem especially in the preweaned age groups. In addition to being less able to tolerate fluid loss caused by diarrhoeal diseases, overwhelming environmental contamination and inadequate maternal and acquired immunity often combine to make this age group more susceptible to these infections (Black, 1985; Radostits, 1985). While several agents including *Escherichia coli*, *Salmonella* spp. and rotavirus have been extensively studied, *Cryptosporidium* has only in recent years received attention as an enteropathogen causing acute diarrhoea in man and animals.

There are three main reasons for the increased attention given to cryptosporidiosis. Firstly, the disease rapidly became known as a complication of host immunodeficiences. For humans the most important of these is caused by infection with the human immunodeficiency virus (HIV) (Soave and Johnson, 1988). Secondly, a number of studies have shown apparent lack of host specificity among mammalian species and this was a feature which set *Cryptosporidium* apart from other coccidia (Tzipori, 1988). Its importance lay in the organism's ability to take advantage of a large number of host species. Thirdly, there is no known effective therapy for cryptosporidial enteritis.

Most of the work done on cryptosporidiosis has dealt with epidemiology, studies of parasite morphology and life cycle, pathogenic mechanisms and diagnosis of the disease. Investigation of treatment and control has been hindered by the

inability to culture the organism *in vitro* and the absence of a symptomatic small animal model. Attempted chemotherapy has included the use of various antimicrobial (Moon, Schwartz, Welch, McCann and Runnels, 1982a; Moon, Woode and Ahrens, 1982b) and antiprotozoal (Angus, Hutchison, Campbell and Snodgrass, 1984) drugs. Improving our knowledge of the parasite's biology may help to develop drugs directed at particular points in the life cycle.

There have been few studies on host immune mechanisms and those published provide some conflicting information. Persistence of infection in acquired immunodeficiency syndrome (AIDS) patients and athymic nude mice (Jaffe, Bregman and Selik, 1983; Heine, Moon and Woodmansee, 1984a) indicated that immunity was T-lymphocyte dependent. It is not understood whether the effector mechanisms of this T-cell dependent immunity are based on antibody, cells or both. While hyperimmune serum against *Cryptosporidium* has been shown to neutralize sporozoite infection of mouse intestine (Riggs and Perryman, 1987), passive lacteal immunity to enteric cryptosporidiosis in mice has been shown to be ineffective (Moon, Woodmansee, Harp, Abel and Ungar, 1988). Both immunocompetent and immunocompromised persons have been shown to be capable of producing specific antibodies to *Cryptosporidium* (Ungar and Nash, 1986). Hence, the role of antibody in cryptosporidiosis is uncertain and definitive studies are needed in this area.

There are three main aims of the work in this study. Firstly, to establish and investigate the infection in a symptomatic laboratory animal model where immunological reagents were readily available. If possible this model would use inbred hosts to facilitate experiments requiring cell transfer between immune and non-immune animals. Secondly, to compare and contrast the laboratory animal model with the infection in young ruminants in terms of parasite biology and immunology. Thirdly, to determine the neutralizing ability of specific immunoglobulins using *in vitro* and *in vivo* techniques.

CHAPTER 1. Cryptosporidiosis, with particular regard to ruminants: a literature review

No attempt will be made to review the literature for all species. Most reports of mammalian cryptosporidiosis in veterinary literature concern the bovine, ovine, caprine and cervine species and indicate that the disease is most important in preweaned animals. Unless otherwise stated, this review will give emphasis to the enteric disease in these species.

1.1. HISTORY AND CLASSIFICATION

Cryptosporidium is a genus in the family Cryptosporiidae, suborder Eimeriina, order Eucoccidiida, subclass Coccidia, class Sporozoa, phylum Apicomplexa (Levine, 1980). The main taxonomic characteristics used to classify *Cryptosporidium* are shown in Table 1.1 (Levine, 1973). The suborder Eimeriina includes the intestinal coccidians *Eimeria* and *Isospora* and the tissue cyst-forming coccidians *Toxoplasma* and *Sarcocystis*.

Cryptosporidium was first described in mice by Tyzzer (1907). Early heterologous cross-transmission experiments using *Cryptosporidium* isolates obtained from guinea pigs (Vetterling, Jervis, Merrill and Sprinz, 1971a) and cats (Iseki, 1979) supported the notion of assigning a new specific name to each additional new animal isolate. By 1985 *Cryptosporidium* had been reported in a wide range of vertebrate hosts (O'Donoghue, 1985) and new species had been proposed without adequate cross-transmission data (Tzipori, 1988).

Numerous reports of heterologous cross-transmission between hosts now indicate that species specificity is probably not a characteristic of most isolates of *Cryptosporidium* (Moon and Bemrick, 1981; Current and Reese, 1986; Klesius, Haynes and Malo, 1986).

There is, however, considerable evidence to indicate that subtle biological differences do exist between isolates of *Cryptosporidium* which account for preferences for a particular host or particular location in the host. Preferential location within the same host was first noticed by Tyzzer in 1910 who, on this basis, proposed two species of *Cryptosporidium* he had found in the mouse. Transmission experiments have shown *C. muris* (Tyzzer, 1910) has a predilection for gastric mucosa while *C. parvum* (Tyzzer, 1912) has a preference for ileal mucosa of the mouse. More recently, cross-transmission experiments to determine host specificity were used as supporting evidence for a proposed new species, *C. baileyi*, infecting chickens (Current, Upton and Haynes, 1986). Similar cross-transmission studies have revealed apparent strain differences or differences in the host specificity of several mammalian and avian isolates of *Cryptosporidium* (O'Donoghue, Tham, De Saram, Paull and McDermott, 1987). Further studies in parasite morphology and host specificity are needed before identifying different species or strains.

Reports of transmission between host species and between sites in the same host may eventually provide supportive evidence for more definitive means of speciation such as antigenic analysis using monoclonal antibodies and iso-enzyme studies. Gel electrophoretic separation of DNA has been used to differentiate isolates of *C. parvum* from *C. baileyi* (Mead, Arrowood, Current and Sterling, 1988).

Table 1.1. Taxonomic classification of *Cryptosporidium*

	Classification	Main characteristics
Phylum:	Apicomplexa	apical complex present; micro-pores present; cilia and flagella absent (except for flagellated microgametes in some groups); all species parasitic.
Class:	Sporozoa	apical complex well developed; reproduction both sexual and asexual; oocysts present; locomotion by body flexion; microgametes flagellated in some groups; monoxenous or heteroxenous life cycle.
Subclass:	Coccidia	mature gamonts small; typically intracellular; mostly in vertebrates.
Order:	Eucoccidiida	merogony present
Suborder:	Eimeriina	macrogamete and microgametocyte develop independently; syzygy absent; zygote not motile.
Family:	Cryptosporidiidae	develop just under surface membrane of host cell; oocysts without sporocysts; with four naked sporozoites; monoxenous; microgametes without flagella.

1.2. HOST RANGE AND SITE OF INFECTION

Infection with *Cryptosporidium* has been reported in a wide range of vertebrate hosts; primates (man, rhesus monkeys, maraques); domestic farm animals (cattle, sheep, goats, pigs, horses, deer); domestic pets (dogs, cats); laboratory animals (mice, rats, guinea pigs, rabbits); domestic fowl and wild birds (chickens, turkeys, quail, pheasant, geese, ducks, parrots, budgerigars, canaries); reptiles (snakes); and fish (compare reviews by Tzipori(1983) and O'Donoghue(1985)). *Cryptosporidium* spp. parasitize a wide variety of epithelial surfaces among these hosts, but those of the gastrointestinal and respiratory tracts are most common.

In naturally acquired and experimentally induced cryptosporidiosis of both the gastrointestinal and respiratory tracts, endogenous stages are located in the microvillous border of infected epithelial cells. In mammals, naturally acquired infection is mainly enteric with parasitism of the abomasum (Anderson, 1987), small and large intestines (Jubb, Kennedy and Palmer, 1985a).

In birds, both respiratory and gastrointestinal tract infections are reported. With enteric infection in birds, parasites occur in small and large intestines with enterocytes of the cloaca and bursa of Fabricius often being infected (Current *et al*, 1986; Lindsay, Blagburn, Sundermann, Hoerr and Ernest, 1986). Respiratory infection in birds can be severe, especially in turkeys, where parasites develop in the microvillous border of the nasopharynx, larynx, trachea, bronchi and air sacs (Lindsay, Blagburn and Hoerr, 1987).

1.3. LIFE CYCLE AND ULTRASTRUCTURE

Many details of the now accepted life cycle were first described by Tyzzer (1910, 1912). Since these reports, most advances have come from ultrastructural studies. There have been four areas of debate since Tyzzer's work : (1) the existence of an oocyst (2) whether the organism should be considered intra- or extra-cellular (3) the number of asexual generations and (4) the importance of thin-walled oocysts. While some issues are still open to interpretation, numerous studies of the life cycle in man, calves, lambs, mice, chickens and cell culture have consolidated an accepted outline similar to other coccidia: asexual followed by sexual endogenous stages, resulting in production of oocysts that can survive outside the host and constitute the infective stage.

Tyzzer in 1910 and 1912, using infected mice, described the oocyst (without sporocysts) containing four naked sporozoites. Vetterling *et al* (1971a) and Vetterling, Takeuchi and Madden (1971b) described two generations of schizont, the first with 8 merozoites and the second with 4, in infected guinea pigs. Even though micro- and macro-gametogony were described, they did not observe oocysts, having examined material taken at 24 hour intervals up to 15 days post inoculation. The second generation schizont was considered to be what Tyzzer (1910) described as a sporulated oocyst. Similarly, Bird and Smith (1980) did not observe oocysts in the lumen of infected human bowel. It is now known that some isolates of *Cryptosporidium*, when transmitted to guinea pigs, have a variable prepatent period ranging from 7-20 days post inoculation with only small numbers of oocysts shed (Angus, personal communication). Pohlenz, Bemrick, Moon and Cheville (1978a), studying cryptosporidiosis in calves, confirmed both the presence of two types of schizont and the existence of oocysts. Oocysts were observed both on enterocytes and in faeces.

Early studies suggested that the parasitophorous envelope, common to all stages, was formed by the parasite and concluded that cryptosporidia were extracellular parasites (Takeuchi, 1971; Pohlenz *et al*, 1978a). Membranes of the parasitophorous vacuole have been shown to be morphologically similar to and continuous with host cell plasma membrane (Hampton and Rosario, 1966; Vetterling *et al*, 1971b; Pearson and Logan, 1983). Marcial and Madara (1986) used electron microscopy and freeze-fracture techniques to further describe the extension of host cell cytoplasm and microfilaments between the inner and outer membranes forming the parasitophorous envelope. This evidence placed the parasite intracellularly, enclosed by host membranes. Ultrastructural studies showed that once inside the host cell, the parasite established contact only with apical cytoplasm through the formation of a complex folded membrane structure referred to as the feeder organelle (Pearson and Logan, 1983; Marcial and Madara, 1986). The relationship of the parasite to the host became acceptably described as intracellular but extracytoplasmic (Goebel and Braendler, 1982).

Current and Reese (1986) described two types of oocyst which differed in the thickness of their walls. Thin-walled oocysts were readily ruptured in the same host releasing sporozoites in what was considered to be an autoinfective cycle. Thick-walled oocysts were structurally similar to the oocysts described in other studies (Pohlenz *et al*, 1978a; Itakura, Nakamura, Umemura and Goryo, 1985). No thin-walled oocysts were seen in studies of *Cryptosporidium* infection in cultured human foetal lung (HFL) cells (Current and Haynes, 1984). They observed that during the first three days post infection, development of endogenous stages in HFL cells was similar to that seen in enterocytes of sucking mice and tissue of chicken chorioallantoic membrane (CAM). From 4 to 8 days post infection the number of endogenous stages increased in mouse and CAM tissues but decreased in HFL cultures. It was suggested that this decline was perhaps due to lack of an autoinfective cycle from thin-walled oocysts. It is

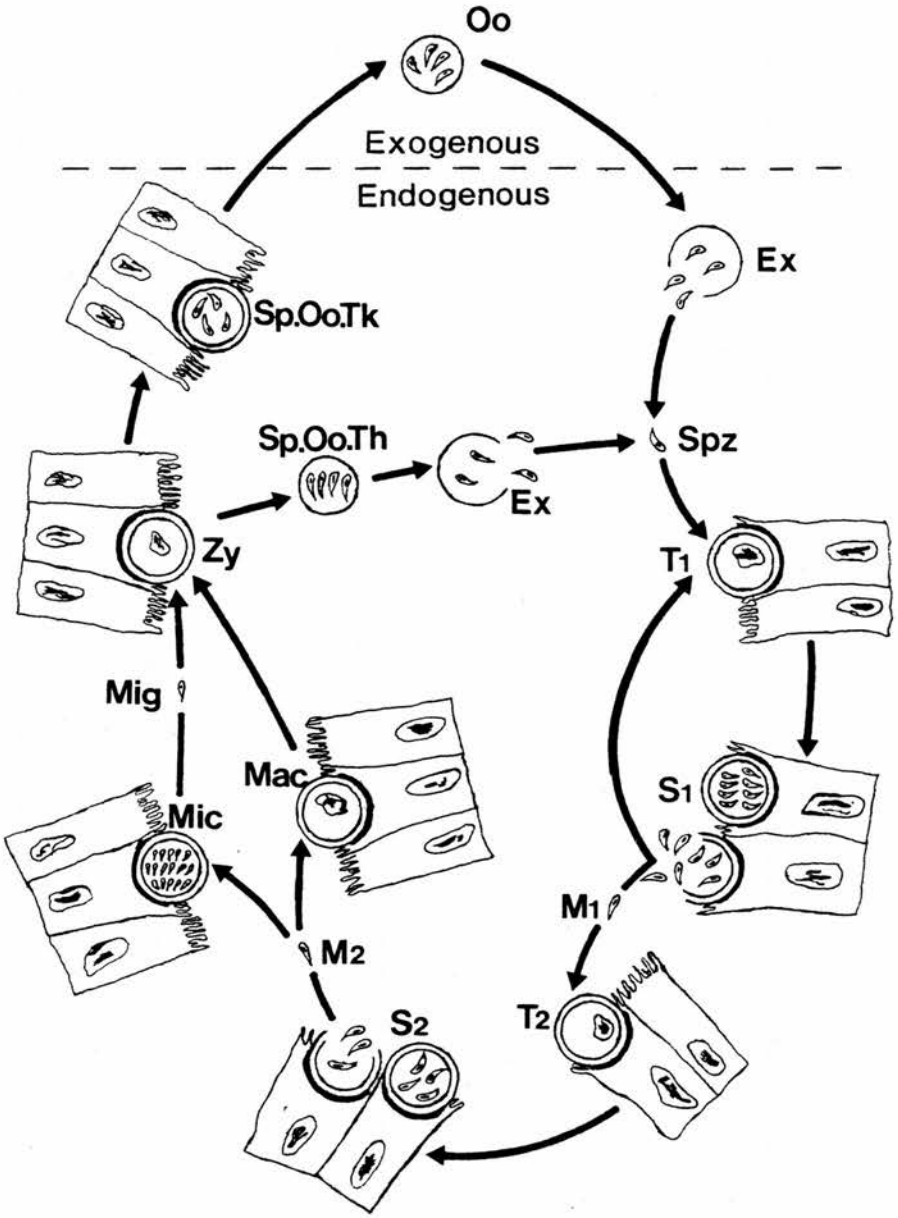
the reinitiation of schizogony from sporozoites released from thin-walled oocysts and a proposed recycling of first generation merozoites (Current and Long, 1983) that enhance the parasite's reproductive potential. A schematic representation of the life cycle, as is understood to occur in intestinal epithelium, is shown in Figure 1.1.

1.3.1. Trophozoites

Sporozoites excyst from ingested oocysts by a process known to be stimulated by exposure to trypsin and bile salts (Current and Haynes, 1984). Just prior to excystation sporozoites exhibit rapid tumbling movements within the oocyst. They are banana shaped with rounded posterior and pointed anterior ends. Flexing and gliding movements combined with extension and contraction of their anterior end are used to probe epithelial microvilli prior to invasion. Merozoites involved in cyclic asexual development invade enterocytes in a similar way. Sporozoites are distinguished from merozoites by the former containing amylopectin granules near the centre of the cell and the latter having a centrally placed nucleus (Current and Reese, 1986). However, Bird and Smith (1980) considered the merozoite nucleus to be in the posterior of the cell.

Sporozoites and merozoites attach to host cell microvilli by a mechanism yet to be determined. What follows is thought to be a process of host membrane invagination and eventual fusion over the top of the organism. This forms a parasitophorous envelope with inner and outer membranes of host origin enclosing the parasite intracellularly in a parasitophorous vacuole (Vetterling *et al*, 1971a,b; Pearson and Logan, 1983; Itakura *et al*, 1985). Adjacent microvilli are displaced laterally, some fusing with the parasitophorous envelope (Pearson and Logan, 1983). Observed vacuolation at the anterior end of sporozoites and merozoites during invasion suggested emptying of rhoptries and/or micronemes; the function and composition of material

Figure 1.1. Schematic representation of the life-cycle of *Cryptosporidium*. Abbreviations: 1 = first generation; 2 = second generation; Ex = excystation; M = merozoite; Mac = macrogamont; Mic = microgamont; Mig = microgamete; Oo = oocyst; S = schizont; Sp = sporulated; Spz = sporozoite; T = trophozoite; Th = thin-walled; Tk = thick-walled.


Oo
Exogenous
Endogenous
Ex
Sp.Oo.Tk
Sp.Oo.Th
Ex
Spz
Zy
T1
Mig
Mac
S1
Mic
M1
M2
T2
S2

released from these organelles is uncertain but may facilitate invagination of the host cell membrane (Current and Reese, 1986). At this stage in the life cycle, the inner envelope membrane still separates the parasite from direct contact with host cell cytoplasm.

After penetration these elongated invasive forms transform into spheroidal trophozoites and dedifferentiate until all organelles are resorbed except nucleus, golgi anlagen, pellicle and cytoplasmic ribosomes (Vetterling *et al*, 1971b). There then follows a series of host and parasite membrane modifications. Firstly, the inner layer of the pellicle regresses over the lateral and lower aspects of the protozoan, leaving only the outer layer or plasma membrane (Marcial and Madara, 1986). The inner membrane normally consists of two unit membranes closely attached to one another (Scholtyseck, 1973). Some researchers consider *Cryptosporidium* merozoites to differ ultrastructurally from those of other coccidia in that the inner membrane of the pellicle does not extend anteriorly into the region of the apical complex (Current and Reese, 1986). Secondly, at the periphery of the zone of attachment, at a level just below that where the parasite indents the terminal web, the inner envelope membrane of host origin and the plasma membrane of the parasite fuse to form a junctional complex (Current and Reese, 1986; Marcial and Madara, 1986). The host membrane, isolated by this fusion and in contact with the parasite, then loses its integrity and a dense band of host microfilaments forms in the adjacent cytoplasm (Current and Reese, 1986; Marcial and Madara, 1986). At this stage the parasite plasma membrane isolated by the fusion zone undergoes extensive folding to form what is referred to as the feeder organelle (Pohlenz *et al*, 1978a).

1.3.2. Schizogony

During formation of the feeder organelle, the rounded trophozoite undergoes marked cytoplasmic proliferation, nuclear

division and infolding of the pellicle to cover each of the divided nuclei and endoplasmic reticulum (Bird and Smith, 1980). This process is called ectomerogony and in the mature schizont, merozoites are attached to a small residual body. The mature merozoite has a two layered pellicle, the inner one composed of two closely attached unit membranes (Marcial and Madara, 1986). Cytoplasmic features observed in merozoites include endoplasmic reticulum with ribosomes, golgi membranes, large nucleus with prominent nucleolus and apical complex (Vetterling *et al*, 1971b; Bird and Smith, 1980). The apical complex is similar to that described for invasive forms of other coccidia but has no apparent micropores or subpellicular microtubules (Current and Reese, 1986).

Some workers (Bird and Smith, 1980; Snodgrass, Angus and Gray, 1984; Itakura *et al*, 1985) thought it possible that just one type of schizont existed which, when mature, contained 8 merozoites. Others (Vetterling *et al*, 1971a; Pohlenz *et al*, 1978a; Current and Reese, 1986) recognized two types of schizont. Type 1, with 8 merozoites, appears first in the life cycle and type 2 follows with 4 merozoites. In both types, merozoites were formed by ectomerogony, budding from a residuum at the base of the parasitophorous vacuole. The continuing presence of abundant type 1 schizonts, over an 8 day post infection period in enterocytes of infected mice and CAM tissues of chicken embryos, led Current and Long (1983) to suggest cyclic development of type 1 merozoites. Similarly, because type 1 schizonts were always more numerous than type 2, over the 3-8 day post infection period, they suggested that merozoites of the latter type proceeded directly into gametogony rather than undergoing cyclic development.

1.3.3. Gametogony

The ultrastructural arrangement of membranes at the parasite-host cell interface during gametogony is similar to

that described for the mature trophozoites. Similarly, gamonts are enclosed within a parasitophorous vacuole (Marcial and Madara, 1986).

Macrogamonts have a large nucleus with no evidence of division and are distinguished by cytoplasm containing clearly defined rough endoplasmic reticulum, amylopectin granules near the parasite's base and wall-forming bodies (Bird and Smith, 1980; Itakura *et al*, 1985). As the macrogamont matures, wall-forming bodies become more numerous in peripheral cytoplasm and 2 types can be distinguished by electron density (Itakura *et al*, 1985; Current and Reese, 1986).

Microgamonts may be difficult to distinguish from macrogamonts before nuclear division has commenced. Microgametogenesis produces 16 nucleated microgametes which bud into the parasitophorous vacuole and surround a large homogeneous residual body. Features of differentiated microgametes include a tubular shape having a tapered posterior and expanded anterior end, a dense nucleus surrounded by endoplasmic reticulum and cytoplasmic microtubules running parallel to the long axis (Bird and Smith, 1980; Itakura *et al*, 1985). They have been observed to attach by anterior ends to only host cell membranes covering macrogamonts which suggested involvement of specific receptors (Current and Reese, 1986).

Fertilization is accomplished by microgamete penetration of the parasitophorous envelope and macrogamont pellicle. Initial contact is probably via a granular adhesion zone at the apical pole of the microgamete (Itakura *et al*, 1985).

1.3.4. Oocyst Formation

Oocyst wall formation begins prior to sporulation. Hence, unsporulated oocysts in parasitophorous vacuoles are often seen to have complete walls and few wall-forming bodies. The wall

consists of 2 layers limited by 3 membranes. Fusion of wall-forming bodies with and emptying of their contents between these membranes results in inner and outer oocyst wall formation (Current and Reese, 1986). The thickness of the outer wall is variable (180-600nm). The inner wall is more uniform (210-255nm) and has a suture which is the site of oocyst wall collapse during excystation.

Sporulation results in formation of 4 sporozoites which are structurally similar to merozoites. The method by which oocysts are finally released from the parasitophorous vacuole has not been studied but in the bowel may simply rely upon degeneration of the envelope after enterocytes are extruded into the lumen. Both thick- and thin-walled oocysts (Current and Long, 1983) have been observed to sporulate while attached to host cells. It is generally the thick-walled oocyst which is detected in faeces while the thin-walled oocyst excysts in the same host.

The prepatent period appears to depend on the host, with some reports as early as 3 days in lambs (Angus, Tzipori and Gray, 1982b; Snodgrass *et al*, 1984) and extending to as long as 14-20 days in hosts such as the guinea pig (Angus, personal communication). Oocysts have been seen in cell culture and on CAM of chicken embryos as early as 3 days post inoculation and in mouse enterocytes on the fourth day post infection (Current and Long, 1983; Current and Haynes, 1984; Current and Reese, 1986). Snodgrass *et al* (1984) described a similar sequence of development for endogenous stages in lambs. There have been reports of oocysts shed as early as 1 or 2 days of age in calves but these were thought possibly to be orally ingested oocysts passing unchanged through the digestive tract (Anderson, 1981).

1.4. EPIDEMIOLOGICAL ASPECTS OF CRYPTOSPORIDIOSIS

Panciera, Thomassen and Garner (1971) and Meisel, Perera, Meligro and Robin (1976) were the first to report *Cryptosporidium* infection in cattle and man respectively. Both reports were of chronic diarrhoea and wasting with a fatal outcome. There are now numerous reports in the literature documenting the infection in wild and domestic animal species and man from many parts of the world.

1.4.1. Occurrence

Cryptosporidium infection appears to be endemic in many populations of domestic stock and is commonly associated with outbreaks of diarrhoea in the young. Surveys of bovine calf diarrhoea in Holland and southern Britain found that *Cryptosporidium* was the second only to rotavirus as the most commonly detected enteropathogen (Reynolds, Morgan, Chanter, Jones, Bridger, Debney and Bunch, 1986; de Visser, Breukink, van Zijderveld and de Leeuw, 1987). A similar calf study in Denmark has shown that infection with *Cryptosporidium* was most frequent in the 4-30 day age group but with a predominance of cases in the 8-14 day age group (Henriksen and Krogh, 1985).

In surveys of calves reared on the farm in which they were born, outbreaks of diarrhoea associated with *Cryptosporidium* were characterised by detection of the first clinical cases in animals 8-12 days of age (Anderson, 1981; Blewett, 1987). Within a week of the first clinical case being detected, there was a rapid rise in prevalence of the infection. As animals were born into the group clinical cases were detected earlier, at 5-6 days of age. The same pattern of spread has been shown in surveys of cryptosporidiosis in red deer calves (Tzipori, Angus, Campbell and Sherwood, 1981b; Blewett, 1987).

Where *Cryptosporidium* was the only agent associated with clinical disease in young ruminants, morbidities between 70-85% have been reported (Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980b; Tzipori, Angus, Campbell and Clerihew, 1981a; Tzipori *et al*, 1981b; Tzipori, Larsen, Smith and Luefl, 1982b). Mortality in such cases tended to be higher in artificially reared lambs and red-deer calves where it was associated with prolonged scour (2-3 weeks) and reduced appetite. In uncomplicated cryptosporidiosis in bovine calves mortality is low but increases when associated with rotavirus or coronavirus infections (Tzipori *et al*, 1980b; Snodgrass and Sherwood, 1983).

1.4.2. Determinants of disease

Host related

Cryptosporidium infection in young ruminants has been shown to produce similar clinical signs as well as distribution and nature of enteric lesions (Tzipori *et al*, 1981b; Heine, Pohlenz, Moon and Woode, 1984b; Snodgrass *et al*, 1984). There are no reports of sex-linked variations in *Cryptosporidium* infection. The majority of reports indicate that clinical disease is associated with the 5-14 days age group. There is an age dependent susceptibility to clinical disease. Lambs and calves infected at 10 and 14 days of age respectively can still develop clinical disease but lambs infected at 20 days showed little or no diarrhoea and 30-day old lambs remained healthy (Pohlenz, Moon, Cheville and Bemrick, 1978b; Tzipori, Angus, Gray, Campbell and Allen, 1981c).

Agent related

Heine *et al* (1984b) speculated that variations in dose and virulence of the parasite probably contribute to variations in clinical signs and lesions. Two important intrinsic factors

contributing to the outcome of infection are reproductive potential of the parasite and the site of parasitism. There is little variation in the site of parasitism in that the parasite always grows within the same part of the host cell and the distribution of enteric lesions has been shown to be similar for many species. In terms of oocyst production, reproductive potential has been shown to vary between isolates (Blewett, 1988). *Cryptosporidium* isolates of bovine, cervine and equine origin, when titrated in sucking mice, were shown to have different reproductive potential at low doses but no difference at higher doses. Hence, virulence may depend both on dose and isolate. Similar findings have been made for other coccidia. For faecal oocyst output to accurately reflect infection by *Eimeria tenella*, the infecting dose should be small to avoid adverse effects on parasite reproduction due to lack of intestinal space (Rose, 1967a; Rose and Long, 1971).

Infection of the intestine with *Cryptosporidium* does not always lead to clinical disease. As shown in reported outbreaks of cryptosporidiosis in bovine and red deer calves, non-diarrhoeic clinically normal in-contact animals may be shedding oocysts (Jerrett and Snodgrass, 1981; Tzipori *et al*, 1981b). There is little evidence to suggest that a state of sterile immunity exists after clinical disease. Instead, *Cryptosporidium* endogenous stages observed histologically in the intestines of normal young adult sheep (Angus, Appleyard, Menzies, Campbell and Sherwood, 1982a) and oocysts recovered from faeces of adult sheep and cattle (Papadopoulou, Xylouri, Mantzious, Spyropoulos and Stoforos, 1988) indicate that a low level carrier status exists in the adult population.

1.4.3. Transmission and maintenance of infection

Oocysts of *Cryptosporidium* were first described by Tyzzer (1910, 1912). Vetterling *et al* (1971a) and Bird and Smith (1980) were not able to confirm the existence of an oocyst in their studies

in guinea pigs and man respectively. There was confusion over the nature of the infective stage and the method of spread of infection. Pohlenz *et al* (1978a,b) had demonstrated that a bovine isolate of *Cryptosporidium* could be transmitted by scrapings of infected ileal mucosa given by gavage. Oocysts were found on epithelial cells as well as in the faeces of infected calves. The concept that horizontal transmission occurred via the faecal-oral route, with the thick-walled oocyst as the infective stage, quickly became accepted.

Except for those reports of calves shedding oocysts at one day of age, there has been little support for the possibility of vertical transmission. Excretion at one day of age has been interpreted as ingested oocysts passing unchanged through the gastrointestinal tract (Anderson, 1981).

It is not known how the viability of *Cryptosporidium* oocysts might change with temperature or other conditions of the micro-environment. Storage of oocysts at 4^{0}C for 4-6 months, either in faeces or a purified state, has not affected viability as shown by the percentage of oocysts able to be artificially excysted and successful animal inoculations (Snodgrass *et al*, 1984; Blewett, personal communication). Reports of outbreaks of diarrhoea attributed to *Cryptosporidium* infection suggest that oocysts might remain viable in the environment for 6-12 months (Tzipori *et al*, 1981b; Angus *et al*, 1982a). The lack of host specificity, shown by many isolates of *Cryptosporidium*, probably enhances survival by not having to depend upon recycling in the same species to ensure that revitalized oocysts contaminate the environment.

Other domestic species and man

The lack of published reports of *Cryptosporidium*-associated diarrhoea in piglets and foals suggests that it is not a problem in these species. Typical clinical signs and enteric lesions

have been described in pigs with experimental crytposporidiosis (Tzipori, McCartney, Lawson, Rowland and Campbell, 1981d). *Cryptosporidium* infections have also been reported in scouring farm piglets but no specific role could be attributed to them (Links, 1982; Sanford, 1987). Reinemeyer, Kline and Stauffer (1984) and Tzipori (1985) did not detect *Cryptosporidium* oocysts in diarrhoeic faeces examined from 66 scouring foals. However, asymptomatic oocyst shedding detected in mares, both before and after parturition, indicate that they may act as a source of the parasite for their foals (Chermette, Boufassa, Soule, Tarnau, Courderc and Lengronne, 1987).

Cryptosporidiosis in man is unusual in that all age groups appear susceptible to clinical disease. There are numerous published reports of cryptosporidiosis in children in day care centres and in adults both with and without obvious professional contact with the infection (Babb, Differding and Trollope, 1982; Blagburn and Current, 1983; Alpert, Bell, Kirkpatrick, Budnick, Campos, Friedman and Plotkin, 1986). Cryptosporidiosis came to the attention of medical scientists along with recognition of HIV infection. Cryptosporidiosis has two distinct clinical forms in man: a self-limiting enterocolitis in immunologically normal persons and a persistent, life-threatening diarrhoea in those immunocompromised, especially through HIV infection. Similar persisting cryptosporidial infections have been reported in Arabian foals with combined immunodeficiency (Snyder, England and McChesney, 1978; Gibson, Hill and Huber, 1983). The pathogenicity of human *Cryptosporidium* isolates for laboratory animals (Current and Reese, 1986) and lambs (Tzipori, Angus, Campbell and Gray, 1982a) indirectly demonstrate the potential zoonotic importance of this parasite.

1.5. ASSOCIATED PATHOGENS

Despite numerous reports of *Cryptosporidium* being associated with diarrhoea in neonatal animals, its specific role is often made obscure by the presence of other known enteropathogens (Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gilette and Baetz, 1978; Snodgrass, Angus, Gray, Keir and Clerihew, 1980; Anderson, 1982; Sanford and Josephson, 1982). Heine *et al* (1984b) and Snodgrass *et al* (1984) showed that *Cryptosporidium*, in the absence of normal intestinal flora or known enteropathogens, could cause diarrhoea in monoinfected, gnotobiotic calves and lambs.

Associated primary enteropathogens include enteric viruses such as rotavirus and coronavirus and bacteria such as enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella* spp.. Infection with ETEC is the most likely cause of diarrhoea in calves and lambs less than 4-5 days of age. All the remaining agents including *Cryptosporidium* must be considered in cases up to 2-3 weeks of age (Jubb *et al*, 1985b). Coronavirus and ETEC can result in high mortalities in untreated calves due to dehydration, acidiosis and hyperkalaemia. In comparison, rotavirus tends to produce a less severe transient diarrhoea (Jubb *et al*, 1985b). Salmonellosis does not always cause diarrhoea and is often associated with calves brought into rearing units (Reynolds *et al*, 1986). In uncomplicated cryptosporidiosis calves tend not to suffer severe dehydration and acidiosis and mortality is low (Tzipori, Smith, Haplin, Angus, Sherwood and Campbell, 1983). All these enteropathogens can often be isolated from the one outbreak. Diagnosis of cryptosporidiosis should take account of other enteropathogens by examining specimens from several infected animals.

Studies of mixed enteric infections in lambs indicate that *Cryptosporidium* induces a more severe disease in lambs aged at least 6 days than ETEC or rotavirus acting alone or in

combination (Tzipori, Sherwood, Angus, Campbell and Gordon, 1981e). While coronavirus-like particles have been detected in the faeces of sheep with transient diarrhoea (Tzipori, Smith, Makin and McCaughan, 1978), the infection is not recognised as an important enteropathogen in this species (McNulty, 1985).

1.6. PATHOGENESIS AND PATHOLOGICAL CHANGES

A common developmental sequence of endogenous stages has been described *in vivo* (for a number of host species) and in *in vitro* studies of the *Cryptosporidium* life cycle (Current and Haynes, 1984; Snodgrass *et al*, 1984; Current and Reese, 1986). Maturation of trophozoites and sexual stages causes disruption and displacement of epithelial cell microvilli (Pohlenz *et al*, 1978b). Ultrastructural degenerative changes evident in the host cell include fine vacuolation and accumulation of dense spherical bodies in apical cytoplasm and disorganisation of microfilaments in the terminal web (Hampton and Rosario, 1966; Heine *et al*, 1984b; Marcial and Madara, 1986). The parasitophorous envelope consists of folds of host cell microvillous membrane. If microvilli facilitate parasite invasion, then this may explain why parasites are rarely reported in the crypts of Lieberkuhn as undifferentiated crypt cells have only sparse, short and irregular microvilli.

Studies in young domestic animals have shown that *Cryptosporidium* can be seen at all levels of the small and large intestine (Tzipori *et al*, 1981b; Angus *et al*, 1982b; Tzipori *et al*, 1983). Proximal small intestine and colon generally carry lower numbers of parasites. No morphological changes are evident up to 48 hours post infection during which time asexual and early sexual generations develop and parasites are often seen in clusters. This has been observed on infected intestinal surfaces and chicken CAM (Angus *et al*, 1982b; Current and Long, 1983; Snodgrass *et al*, 1984).

At 3 days post infection, all developmental stages are present and associated with widespread damage. It is not known how many repetitions of the asexual cycle occur, if mucosal damage is equally attributable to the asexual and sexual cycles, or if these factors vary between host species to account for varying expression of clinical signs.

Histopathological changes are similar for most species of young animals including laboratory rodents (Tzipori *et al*, 1981b; Angus *et al*, 1982b; Heine *et al*, 1984a,b). Low columnar or cuboidal cells with disordered nuclei cover atrophic villi. Villous extrusion zones are more obvious with aggregates of cells in various stages of degeneration, often with attached endogenous forms. In calves and lambs widespread villous fusion has been described with epithelial bridges between contiguous villi. Endogenous forms can be seen all over the villi but only occasionally in the crypts of Lieberkuhn. Small intestinal crypts have been described as hypertrophic and/or hyperplastic with increased numbers of cells containing mitotic figures. Villous lamina propria and epithelium are usually infiltrated by polymorphonuclear leucocytes and mononuclear cells including macrophages, lymphocytes and plasma cells. In the large intestine endogenous forms are seen both on surface and gland epithelium and a similar inflammatory cell infiltrate is present. Goblet cell numbers are reduced in small and large intestine during the infection.

Atrophic villi and hypertrophic crypts are seen in a wide variety of enteric conditions. This type of lesion can develop by two mechanisms : 1. initial loss of damaged epithelial cells with contraction of villi, followed by compensatory hypertrophy of crypts; 2. initial crypt hypertrophy followed by premature sloughing of the new cells which fail to differentiate (Jubb *et al*, 1985c). In both lesions villi are eventually covered by poorly differentiated cuboidal or low columnar epithelium. In

the first, surface erosion may be evident where there is insufficient epithelium to cover damaged areas. In the second, crypt hypertrophy is the early and outstanding change consistently present. Here, damage to the villous epithelium is not always a prerequisite and crypt hypertrophy is thought to be induced by cell-mediated immune events in the mucosa. Restoration of normal mucosal topography occurs within a few days or several weeks dependent on whether the crypt hypertrophy was in response to villous damage or primarily due to cell mediated(CM) immunity (Jubb *et al*, 1985c).

Study of *E. nieschulzi* in athymic nude rats and their heterozygous litter mates has indicated that the villous atrophy observed was largely thymus dependent (Rose and Hesketh, 1982). In contrast, T-cells do not appear to be essential in the development of lesions in athymic mice with cryptosporidiosis (Heine *et al*, 1984a). Studies of histopathological change during cryptosporidiosis have not suggested that villous atrophy was preceded by crypt hypertrophy. Hence, direct damage by the parasite may be most important in the development of lesions. However, a study of sequential change to villus-crypt architecture from the time of infection to its resolution has not been carried out for cryptosporidiosis.

Electron microscope studies indicate that the feeder organelle and base of the parasitophorous vacuole remain attached to the host cell after release of endogenous stages (Pohlenz *et al*, 1978a; Current and Reese, 1986). However, old attachment sites on the surface of apparently intact cells may represent sites of organisms removed either by natural shedding or specimen processing. The extent of damage to the host cell caused by development and release of endogenous stages has not been studied thoroughly. At the very least, increased cell fragility might be expected but it is not certain that these cells are prematurely lost from the villus. It is this initial damage to and accelerated loss of absorptive epithelium that

must be demonstrated to be certain that crypt hypertrophy is in response to villus atrophy.

Malabsorption and maldigestion, hypersecretion by an intact mucosa, and mucosal exudation via increased capillary and/or epithelial permeability are three mechanisms by which primary enteric disease may lead to diarrhoea (Moon, 1983).

It has been shown in both conventional and gnotobiotic lambs and calves (Angus *et al*, 1982b; Heine *et al*, 1984b; Snodgrass *et al*, 1984) that infection with *Cryptosporidium* results in partial atrophy, fusion and distortion of villi. These lesions probably contribute to malabsorption due to diminished villous surface area and number of absorptive cells (Tzipori, 1988). Maldigestion and malabsorption may be evident before villous atrophy is apparent in cryptosporidiosis because early disruption to absorptive cell microvilli probably reduces membrane-bound enzymes. Tzipori *et al* (1981e, 1983) demonstrated reduced membrane-bound lactase activity in distal portions of lamb and calf small intestine damaged by *Cryptosporidium*. Lactase activity in calves was also shown to be reduced in more proximal segments where infection and mucosal damage were considered minimal.

Diarrhoeal diseases arising from hypersecretion include those caused by ETEC and *Vibrio cholerae*. Enterotoxins produced by these bacteria cause hypersecretion of water and electrolytes from a mucosa which is structurally unaltered. Supportive evidence is lacking for the production of toxins of this type by *Cryptosporidium*.

An excess of unabsorbed bile acids in the colon may result from ileal damage. Bile acids are known to cause mild damage to colonic epithelium and to stimulate cyclic-3'5'-adenosine monophosphate-mediated secretion of fluid and electrolytes from colonic mucosa resulting in diarrhoea (Jubb *et al*, 1985d).

Whether this mechanism is significant to *Cryptosporidium* induced diarrhoea has not been examined.

Exudation of serum proteins into the intestinal tract during infectious diarrhoeal disease has been described in calves (Marsh, Mebus and Underdahl, 1969). It is not known whether mucosal inflammation induced solely by *Cryptosporidium* infection can result in sufficient vascular and epithelial permeability changes to distort the normal serum albumin/globulin ratio.

1.7. RESISTANCE TO *CRYPTOSPORIDIUM* INFECTION

There are several reports in the literature which indicate that immunity does influence the outcome of *Cryptosporidium* infection. *Cryptosporidium* is rarely associated with neonatal equine scour (Reinemeyer *et al*, 1984; Tzipori, 1985; Chermette *et al*, 1987). In contrast, Arabian foals with combined immunodeficiency have been reported to suffer severe *Cryptosporidium* infection (Snyder *et al*, 1978; Gibson *et al*, 1983). Similarly, other genetic (congenitally athymic nude mice), infectious (AIDS resulting from HIV infection) or drug induced (cyclophosphamide) immunodeficiencies are associated with persistent cryptosporidiosis (Heine *et al*, 1984a; Rehg, Hancock and Woodmansee, 1987; Soave and Johnson, 1988). *Cryptosporidium* infection is transient in normal immunocompetent humans and animals (Sherwood, Angus, Snodgrass and Tzipori, 1982; Tzipori *et al*, 1983; Soave and Johnson, 1988).

1.7.1. Passively acquired immunity

Observations from field outbreaks of *Cryptosporidium* associated diarrhoea neither support nor deny a protective role for colostral antibody. Higher morbidity and mortality has been described for *Cryptosporidium* associated diarrhoea in artificially reared neonates and those born to inexperienced

dams (Snodgrass *et al* 1980; Tzipori *et al* 1981a; Angus *et al* 1982a). However, there has been no field study of susceptibility to cryptosporidiosis in relationship to specific passively acquired antibody.

Neonatal piglets and ruminants lose the capacity to absorb large quantities of colostral antibody into the circulation after approximately 36 hours of life (Lecce and Morgan, 1962). Hence, an investigation of colostral antibody protection must consider the role of antibody remaining unabsorbed and that which is secreted back into the lumen of the intestine. In bovine mammary secretions there is a rapid fall in immunoglobulin content over the first few days. Although functional significance may not be related to abundance, it is interesting to relate this decline to the far greater occurrence of cryptosporidiosis in neonatal ruminants compared to piglets. During the first 3 days of bovine lactation the levels of all immunoglobulin classes (IgG_1, IgG_2, IgM and IgA) fall rapidly. By 4 days after parturition the concentration of each immunoglobulin class has been found to be less than approximately 1mg/ml of milk (Porter, 1972). While IgM and IgG have a similar rate of decline in the porcine lactation, that of IgA is not as marked (Porter, Noakes and Allen, 1970). After the first 4 days IgA becomes the predominant immunoglobulin in sow milk, remaining at a level of approximately 10mg/ml during the first 4 weeks of lactation (Porter *et al*, 1970). Hence, enteric protection by unabsorbed lacteal antibody may be short-lived in ruminants compared to pigs.

The major immunoglobulin of ruminant colostrum and post colostral serum is IgG_1, accounting for approximately 80% of the total (Porter, 1972). The half-life of bovine postcolostral IgG_1 is approximately 18 days while that of IgM and IgA is 4 and 2 days respectively (Macdougall and Mulligan, 1969; Porter, 1972). In lambs, similar figures have been found for IgM and IgA but IgG was found to have a half-life of approximately 14 days

(Smith, Wells, Burrells and Dawson, 1976). Although IgM, IgA and IgG are all secreted into the intestines of cattle and sheep (Porter, Noakes and Allen, 1972; Cripps, Husband and Lascelles, 1974; Husband and Lascelles, 1974), the greater quantity and longer half-life of postcolostral IgG may make it a better candidate for protecting against neonatal cryptosporidiosis. In young calves the clearance of serum IgG_1 can mostly be accounted for by transfer into the intestine (Besser, McGuire and Gay, 1987).

Compared with normal calves, those with enteritis have a greater rate of loss of colostrum-derived immunoglobulins and other serum proteins into the intestines (Macdougall and Mulligan, 1969; Marsh *et al*, 1969; Fisher, Martinez, Trainin and Meirom, 1975). Secreted IgA has been found to be associated with the mucus layer over the intestine and this may enhance its persistence (Porter *et al*, 1972). If function is retained, secretion of colostrum-derived antibody could be important until active immunity develops.

Specific antibody to surface epitopes may function to block attachment receptor sites on motile stages of *Cryptosporidium*. This role for antibody was suggested in the work of Riggs and Perryman (1987). Heat-inactivated hyperimmune bovine serum against *Cryptosporidium parvum* sporozoites neutralized their capacity to infect sucking mice. Preimmune serum with low antibody titre did not prevent infection. Whether increased antibody titre was responsible for protection or some other non-immunoglobulin component enhanced by the immunization schedule was not investigated. Specific colostral IgG and IgA provided little or no protection against infection in sucking mice, however, this may have been due to low antibody titre (Moon *et al*, 1988).

1.7.2. Actively acquired immunity

The kinetics and specificity of the endogenous antibody response has not been investigated in relationship to oocyst shedding.

For a wholly enteric infection, presentation of antigen to gut associated lymphoid tissue would be expected as the first step in mounting a specific immune response. *Cryptosporidium* antigen, both in a degraded form and as morphologically recognizable parasites, has been described deep within M cells overlying Peyer's patches and in subjacent macrophages (Marcial and Madara, 1986; Landsverk, 1987).

Both immunofluorescent and enzyme immuno-assays have been used to detect antibody against *Cryptosporidium* in sera from animals and both immunocompetent and immunocompromised humans (Tzipori and Campbell, 1981; Campbell and Current, 1983; Ungar, Soave, Fayer and Nash, 1986; Casemore, 1987). These studies indicate that exposure to or infection with *Cryptosporidium* is common both in human and animal populations.

It is not known which *Cryptosporidium* antigens are involved in protective immunity or which stages in the life cycle carry them. Investigation of *Cryptosporidium* antigens using electrophoretic separation and immunoblotting procedures has received little attention. Blots of disrupted oocyst antigen reacted with convalescent calf serum have detected nine bands with molecular weights greater than 60Kd. (Lazo, Barriga, Redman and Bech-Nielsen, 1986). They reported poor transfer of protein near the gel origin and this may have prevented other bands being detected. Ungar and Nash (1986) showed that most people infected with *Cryptosporidium* produce antibodies which recognize a 23Kd. antigen.

Studies on immunity to other enteric coccidia have shown a dependence on the presence of functional T-cells. Comparisons

made between normal and congenitally athymic (nude) mice or rats have indicated: a primary role for "effector" T-lymphocytes in immunity to *E. falciformis* var. *pragensis* in mice (Mesfin and Bellamy, 1979) and a thymic dependence for the architectural changes and cellular infiltrations seen in the intestines of *E. nieschulzi* infected rats (Rose and Hesketh, 1982; Huntley, Newlands, Miller, McLauchlan, Rose and Hesketh, 1985). Adoptive transfer of immunity to *E. vermiformis* infection in mice was accomplished with dividing mesenteric lymph node cells (Rose, Wakelin, Joysey and Hesketh, 1988). Recipients developed earlier and sometimes higher serum titres of specific antibodies but there appeared to be no correlation between these titres and protection. Antibody titres in intestinal secretions were not examined. This, together with characterization of the cells transferring immunity, is needed before deciding the relative contributions of B- and T-lymphocytes to immunity.

The role of antibody secreted into the intestine has been examined with *Giardia muris* infections in mice. This infection is cleared by normal mice where IgA and IgG bind to trophozoites in the intestine. Athymic mice showed little evidence of this and became chronically infected (Heyworth, 1986). Similarly, immunity to *E. falciformis* infections in mice was associated with production of sporozoite specific IgA detected in intestinal mucus (Douglass and Speer, 1985). Congenitally athymic (nude) mice have a reduced ability to produce IgG_1, IgG_2 and IgA (Haaijman, Slingerland-Teunissen, van Oudenaren, Mink and Benner, 1980). Hence, while experimentation using athymic rodents may provide supportive evidence for T-cell dependent immunity, it may not distinguish between their helper-inducer and cytotoxic-suppressor functions.

A primary role for antibody in enteric protozoan infection was apparent in work describing: passive protection of chickens against *E. tenella* infection using monoclonal antibody (Crane, Murray, Gnozzio and MacDonald, 1988) enhanced neutrophil and

macrophage adherence and phagocytosis of *G. muris* trophozoites in the presence of specific IgA and IgG (Kaplan, Uni, Aikawa and Mahmoud, 1985).

The relative importance of antibody and cell-mediated immunity in cryptosporidiosis has been given little attention. Similarly, the role of intestinal mucus and of antibody associated with it has not been studied.

1.7.3. Age-related resistance

Natural and experimental infections indicate that diarrhoea due solely to *Cryptosporidium* would not be expected beyond 30 days of age in normal calves (Anderson, 1981; Moon and Bemrick, 1981; Henriksen and Krogh, 1985). Other studies have shown a similar age susceptibility in lambs (Tzipori *et al*, 1981c; Anderson, 1982). Endogenous stages of *Cryptosporidium* observed histologically in the intestines of normal young adult sheep (Angus *et al*, 1982a) and oocysts recovered from faeces of adult sheep and cattle (Papadopoulou *et al*, 1988) indicate that a low level carrier status exists in the adult population. Hence, resistance in adult animals may relate only to clinical disease.

Study of resistance, related only to age, requires the use of experimental animals which have not been exposed directly to *Cryptosporidium* antigens or indirectly via antigens perhaps shared with other coccidia and microflora. These conditions can best be met by using specific pathogen free rodents as experimental animals.

Age-related resistance does not appear to be influenced by T-cell deficiency. Infection of either normal or athymic mice at 42 days of age produces only mild subclinical infection with no histological changes observed in the intestinal mucosa (Heine *et al*, 1984a). The mechanism for age-related resistance operating in the gastrointestinal tract does not apply to other mucosal

systems since patent infection is able to establish in uterine epithelium of adult immunocompetent mice (Liebler, Pohlenz and Woodmansee, 1986). Similarly, it does not solely depend upon the presence of a normal intestinal microflora. Normal adult conventional mice remained resistant to *Cryptosporidium* infection after treatment with antibiotics designed to deplete intestinal microflora (Harp, Wannemuehler, Woodmansee and Moon, 1988).

Innate age resistance to clinical disease may come from an ability to avoid severe infection of the small intestine. In this way malabsorption, resulting from villous atrophy, would not cause overloading and increased lumenal osmolarity in the large intestine that resulted in neonatal diarrhoea. How the population dynamics of various endogenous stages might influence the outcome of infection has not been studied. Physiological conditions in the adult small intestine might not be optimal for synchronous excystation and localized infection. Spread of infection along a much expanded adult small intestine may interfere with mechanisms governing recycling of asexual generations and sexual reproduction.

Changes in gastrointestinal tract structure and physiology occurring during and after weaning perhaps warrant further attention to understand their influence on innate age resistance.

1.8. INVESTIGATIVE LABORATORY TECHNIQUES

1.8.1. Diagnosis

Direct demonstration of the parasite

Histological examination of infected tissues, obtained as a biopsy or at necropsy, and microscopic examination of faecal

material provide reliable means of diagnosis. The mere detection of endogenous or exogenous stages by these methods is not diagnostically significant. Large numbers of endogenous or exogenous stages, in association with typical clinical signs and enteric lesions, would constitute a diagnosis in the absence of other complicating enteropathogens. Because *Cryptosporidium* infection can be subclinical or associated with other enteropathogens, its distribution in healthy animals should be considered in interpreting results (Moon *et al*, 1978; Bulgin, Anderson, Ward and Evermann, 1982).

Tissue autolysis, resulting in dislodgement of parasites from cells (Pohlenz *et al*, 1978b), and choice of stain (Gibson *et al*, 1983) can influence histological diagnosis. Similarly, choice of stain and use of various oocyst concentration techniques can influence microscopic diagnosis. Oocysts can be detected in smears of faecal material by direct staining with: Giemsa (Anderson, 1981), carbol-fuchsin (Angus, 1987), methylene blue-eosin (Cross and Moorhead, 1983), phenol auramine (Nichols and Thom, 1984) or by negative staining with nigrosin (Pohjola, 1984). Concentration by flotation can be done using saturated sugar (Anderson, 1981) or salt solutions (Willson and Acres, 1982; O'Donoghue, 1985).

Indirect demonstration of the parasite

Methods include demonstration of specific antibodies and inoculation of infected host material into laboratory animals. Specific antibodies have been widely detected in healthy and unhealthy animals from several species (Tzipori and Campbell, 1981; Campbell and Current, 1983). The lack of information on kinetics of endogenous antibody response, together with poor correlation to clinical disease and the likelihood of confounding maternal antibody in neonatal animals make serological diagnosis complicated. The use of laboratory animals suffers from variations in parasite infectivity

(Blewett, 1988) and host susceptibility (Angus, Hutchison and Munro, 1985). The variability and lack of interlaboratory standardization associated with indirect diagnostic tests could make them unsuitable for routine use.

1.8.2. Purification of oocysts and excystation

The degree of purity required depends on how the inoculum is intended to be used. Successful animal infections can be obtained using faecal homogenates as inocula (Tzipori *et al*, 1981c). Purified and quantitated inocula are required for monoinfection of gnotobiotic animals or cell cultures (Current and Haynes, 1984; Heine *et al*, 1984b).

Initially faeces are diluted and homogenized in water, then passaged through a graded series of sieves to separate oocysts from large particulate debris (Anderson, 1981; Heine *et al*, 1984b). Incorporating either ethyl acetate (1:10 dilution) or ether in the faecal homogenate can aid in extracting fat and allows better dispersion of oocysts (Luft, Payne, Woodmansee and Kim, 1987; Riggs and Perryman, 1987). Washing and differential centrifugation (500-1400g, 10min) of oocysts in water then allows finer debris to be discarded in the supernate (Blewett, personal communication). However, if oocysts are concentrated in this way, then inevitable loss in the supernate must be balanced against required purity. Alternatively, oocysts can be concentrated by flotation in saturated sugar or salt solutions (Anderson, 1981; Ungar *et al*, 1986). Sucrose and Percoll discontinuous gradient centrifugation have also been used for this purpose (Waldman, Tzipori and Forsyth, 1986; Luft *et al*, 1987).

Bacteria-free oocysts can be obtained by decontamination with 60% ethanol (Tzipori *et al*, 1983), 2.5-3.2% peracetic acid (Heine *et al*, 1984b; Harp *et al*, 1988) or by using antibiotics (Current and Haynes, 1984; Snodgrass *et al*, 1984).

Cryptosporidium sporozoites have been obtained by *in vitro* excystation using a technique developed for *Sarcocystis* sporocysts (Current and Long, 1983). Excystation was a two-step procedure with oocysts first being treated with a reducing agent (0.02M cysteine HCL) then incubated under CO_2; secondly oocysts were exposed to a trypsin-bile salt mixture (Box, Marchiondo, Duszynski and Davis, 1980). In contrast to *Eimeria*, *Isospora* and *Sarcocystis*, *Cryptosporidium* oocysts have been shown to excyst in the absence of reducing conditions or exposure to a trypsin-bile salt mixture. However, the latter did enhance the process when incubation was at 37^0C and pH 7.6 (Fayer and Leek, 1984).

Exposure to a trypsin-bile salt mixture and incubation at 37^0C were confirmed as stimuli for excystation of *Cryptosporidium* oocysts (Reduker and Speer, 1985). The presence of bile salts and trypsin appeared to have a beneficial effect on maintenance of sporozoite integrity as judged by deterioration of free sporozoites in their absence (Reduker and Speer, 1985). When oocysts were incubated in excystation media (0.25% w/v trypsin and 0.75 w/v sodium taurocholate in Hank's balanced salt solution) at 37^0C, approximately 50% of oocysts excysted after 90 minutes without deterioration of free sporozoites (Reduker and Speer, 1985).

1.8.3. Propagation in cell culture and chicken embryos

Complete development (from sporozoite to sporulated oocyst) of human and bovine isolates of *Cryptosporidium* has been demonstrated in chicken embryos (Current and Long, 1983), cultured HFL cells and primary chicken kidney and porcine kidney cells (Current and Haynes, 1984).

During the first 3 days after infection development of endogenous stages in HFL cells was similar to that seen in

enterocytes of sucking mice and tissue of chicken CAM (Current and Long, 1983; Current and Haynes, 1984). From days 4-8 post infection the number of endogenous stages increased in mouse and CAM tissues but decreased in HFL cultures. It was suggested that this decline in reproductive potential was due to the absence of an autoinfective cycle from thin-walled oocysts and that most thick-walled oocysts were not released from parasitophorous vacuoles into the culture medium. It is this reduced reproductive potential of *Cryptosporidium* in cell culture that necessitates the use of small domestic ruminants and laboratory animals to maintain stocks of oocysts for experimentation.

The use of cell culture methods would be useful in testing potentially therapeutic drugs, however this may depend on using cell lines where vigorous infections can be established.

1.9. TREATMENT AND CONTROL

Neonatal animals suffering from debilitating diarrhoea, caused by *Cryptosporidium* infection, are treated palliatively with rehydration therapy and intestinal absorbents and may be given antimicrobial agents (Tzipori *et al*, 1980b). Surface disinfectants are available for farm buildings and laboratories (Campbell, Tzipori, Hutchison and Angus, 1982) but specific chemotherapy is not available for parenteral or oral administration.

Remission of cryptosporidiosis has been reported in a child with congenital agammaglobulinaemia after treatment with hyperimmune bovine colostrum administered both orally and by nasogastric tube (Tzipori, Robertson and Chapman, 1986). However, in other human cases oral administration of bovine colostral anti-*Cryptosporidium* antibody has failed to alter the course of the infection (Saxon, 1987).

A variety of antimicrobial and antiprotozoal agents has been tested in calves (Moon *et al*, 1982b), pigs (Moon *et al*, 1982a) and mice (Angus *et al*, 1984) without success. As greater understanding of the parasite's biology and composition become available, particularly from studies of molecular biology, drugs may emerge which are able to target vulnerable points in the life cycle. The need for such drugs is nowhere more evident than in cases of persistent *Cryptosporidium* infection in AIDS patients where attempts at therapy have been similarly unsuccessful (Soave and Johnson, 1988).

1.10. FEATURES OF INTESTINAL IMMUNITY IN NEONATAL LAMBS AND RATS

As part of the work undertaken, a symptomatic small animal model for study of cryptosporidiosis was developed in neonatal rats. This enabled a pathological and immunological comparison to be made between cryptosporidiosis in neonatal rats and lambs (both less than 15 days of age). Therefore, it is relevant to compare the immunocompetence of these hosts, as far as it relates to enteric disease, during the neonatal period.

1.10.1. Immunoglobulin absorption

Lamb

Neonatal ruminants do not absorb immunoglobulin across the placenta and are born agammaglobulinaemic. Over approximately the first 36 hours of life they non-selectively absorb into the circulation macromolecules and all classes of immunoglobulin (Lecce and Morgan, 1962; Halliday, 1965; Klaus, Bennet and Jones, 1969). This is accomplished by a pinocytotically active epithelium in the lower small intestine (Hardy, 1969). It is not known whether cessation of macromolecular uptake (closure) occurs because this epithelium is replaced or because it loses

the ability to transport this material out of the cell into the tissue. Closure is not associated with a marked acceleration of epithelial cell replacement during the neonatal period (Moon and Joel,1975).

Rat

Foetal rats receive some maternal antibody via the yolk sac but in addition selectively absorb IgG from milk in the duodenum and jejunum (Rodewald, 1973). Selectivity is accomplished by enterocytes having receptors specific for the Fc portion of IgG (Borthistle, Kubo, Brown and Grey, 1977; Morris, 1980). Non-selective absorption of macromolecules occurs in the rat ileum. In contrast to selective absorption in the proximal small intestine, protein absorbed in the ileum is subjected to lysosomal digestion (Cornell and Padykula, 1969). Some degradation of immunoglobulin may also occur with nonselective absorption in newly-born lambs since lysosomes seem to be involved in transferring them across the intestinal cells (Healy, 1977). Closure in the rat small intestine occurs after replacement of neonatal epithelium by cells unable to absorb macromolecular substances (Clarke and Hardy, 1969). Intestinal epithelial cell replacement in rats is slow at birth but accelerates markedly over the neonatal period (Koldovsky, Sunshine and Kretchmer, 1966).

1.10.2. Ontogeny of specific antibody response

Lamb

The lamina propria of a lamb's intestine expands soon after birth with increasing numbers of lymphoid cells which include plasma cells by 10 days of age (Reynolds, 1980). Although the onset of humoral immune competence is associated with surface immunoglobulin positive ($_sIg^+$) lymphocytes from the spleen, the major source of B-lymphocytes in the sheep are the Peyer's

patches (Reynolds, 1985). Although large numbers of $_{s}Ig^{+}$ lymphocytes are produced in primary follicles of Peyer's patches from 85 days gestation, their proportion of the circulating pool remains constant until birth. This changes in the first weeks of life with the proportion of $_{s}Ig^{+}$ lymphocytes in blood and lymph increasing from 3-4% to 25-30% of the total population (Al Salami, Simpson-Morgan and Morris, 1985). The role of Peyer's patches in providing $_{s}Ig^{+}$ lymphocytes is largely complete by 1 month of age; they reach maximum size in 3-month old lambs and involute by 15 months (Reynolds, 1980). The sheep foetus can produce specific antibody against a variety of injected and orally administered antigens from approximately 65-70 days gestation; the first antibody produced is IgM; IgG is not produced until 90 days gestation (Fahey and Morris, 1978).

Lambs immunized orally 6-15 days before birth with horse spleen ferritin produced specific IgM antibody in the serum while both IgM and IgA were associated with intestinal secretions (Husband and McDowell, 1975).

Protective immunity was induced prenatally in lambs orally immunized 14 days before term with a killed *Salmonella typhimurium* vaccine (Husband and McDowell, 1978). These experiments have demonstrated early immunocompetence associated with intestinal lymphoid tissues in the lamb.

Rat

Peyer's patches are not microscopically visible until 2 days before birth and lymphoid aggregates in the large intestine do not start to develop until after birth (Hummel, 1935). Peyer's patches at 4 days of age consist mainly of $_{s}Ig^{+}$ lymphocytes of the IgM class and numerous T-lymphocytes forming a broad band near the serosal side; twelve days after birth Peyer's patches resemble those of adults with primary follicles and

interfollicular areas occupied mainly by IgM positive cells and T-lymphocytes respectively (Wilders, Sminia and Janse, 1983). The earliest secondary follicles were seen in Peyer's patches in the proximal small intestine at approximately 22 days of age (Hummel, 1935). The number of macroscopically visible Peyer's patches and their sizes increase with age up to 6 weeks. Furthermore they do not involute in adult life (Hummel, 1935).

As weanlings (21-35 days of age), the amount of IgA antibody found in conventional rat intestinal perfusates was 2.3μg/ml; after weaning the amount of IgA rose to adult levels, approximately 50μg/ml at 90-120 days (Ebersole, Smith and Taubman, 1985). Similar studies with mice have shown a comparable increase in IgA antibody levels (Van der Heijden, Bianchi, Stok and Bokhout, 1988). In the small intestine, as in mesenteric lymph node and Peyer's patches, the number of immunoglobulin secreting cells is very low before and at weaning. After weaning (21-42 days), there is a rapid increase in the number of IgA secreting cells (Van der Heijden *et al*, 1988). Investigation of the effect of neonatal thymectomy on the level of salivary and serum immunoglobulins in rats has indicated that their ability to produce secretory IgA responses is thymus dependent (Ebersole, Taubman and Smith, 1979; Ebersole, Taubman, Smith and Frey, 1982).

Dendritic cells which are positive for the Class II Ia major histocompatibility complex antigen (Ia^+) are first seen in large numbers in the gut wall of foetal rats at 16 days gestation, 5 days before the first B- and T-lymphocyte could be distinguished (Wilders *et al*, 1983). Ia^+ dendritic cells which accumulated in structures recognizable as Peyer's patches from 20 days gestation differentiated into cells with characteristics of antigen presenting cells (Wilders *et al*, 1983; Van Rees, Dijkstra, Van Der Ende, Janse and Sminia, 1988). By 12 days of age Ia^+ dendritic cells are situated between epithelial cells of Peyer's patch dome areas, just beneath this epithelium, and in

interfollicular T-cell regions (Wilders *et al*, 1983). Despite the early appearance of these cells, gut associated lymphoid tissue in neonatal rats does not appear to be functionally mature until several days after birth. This may influence the response of neonatal rats to foreign antigens entering the intestine. Peyer's patches have been shown to have an important function in regulating intestinal immune responses in rats (Enders, Ballhaus and Brendal, 1988).

There appear to be no studies comparing the kinetics of specific antibody production in neonatal rats and lambs. The above discussion indicates that, at birth, the lamb is in a more advanced state of humoral immunocompetence.

1.10.3. Ontogeny of cell-mediated(CM) immunity in the intestine

Lambs

Because sufficiently inbred or genetically identical sheep have not been readily available for study, the role and maturity of CM immunity in the first weeks of life has received little attention.

Rats

The availability of highly inbred strains of rats has enabled study of CM immunity in this species by adoptive transfer of immune cells. While CM immunity has been studied in neonatal rats with helminth infections, information is lacking on enteric protozoal diseases in this age group.

CHAPTER 2. Materials and methods

2.1. INTRODUCTION

This chapter provides details on materials and methods applicable to all experiments conducted in this study. Details specific to individual experiments are given at appropriate points in the text.

2.2. LABORATORY ANIMALS

2.2.1. Rodents

Three strains of mouse (randomly bred Swiss White and inbred CBA and Porton WB) and three strains of rat (randomly bred Wistar and inbred F344 and Lister) were used in this study. Pregnant females were housed individually, given pelleted food (irradiated rat and mouse breeding diet) and water ad libitum, and allowed to whelp and suckle their pups. Pups were weaned at 21 days of age. All the mice and rats used in this study were raised free of *Cryptosporidium* spp.

Cages containing principals and controls were housed in the same room. Controls were cared for, observed and sampled before any of the principals.

2.2.2. Ungulates

Oocyst donors

Conventional male calves and lambs, obtained within 12-24 hours of birth, were allowed colostrum and then moved to rooms in isolation facilities. They were fed a milk replacer diet 2-4 times daily until approximately 21 days of age.

Experimental colostrum deprived lambs

Scottish Blackface-cross lambs were taken at birth, deprived of colostrum and housed in rooms in an isolation facility. Principals and controls were housed separately. Prior to introducing the lambs, the rooms had been steam cleaned. Final decontamination was by fumigation with a 35% ammonia solution (sp. gr., 0.88) which is known to destroy *Cryptosporidium* oocyst infectivity (Campbell *et al*, 1982). Staff changed clothes before entering the rooms and access to the lambs was via a disinfectant foot-bath containing 5% iodophore.

All lambs were treated with antibiotics within 15 minutes of birth using Oroject N/S (Willows Francis Veterinary) per os and intravenous Trivetrin (Wellcome). They were treated with Terramycin/LA (Pfizer), given intramuscularly, and Oroject N/S per os at 2 and 4 days of age after which antibiotic cover was discontinued. Using sterile technique blood samples were taken for bacteriology from the jugular veins of 7 lambs at 1 day of age. Blood was cultured aerobically and anaerobically at 37^{0}C for 24-48 hours using 5% sheep blood agar (Gibco) and MacConkey agar (Oxoid).

Colostrum deprived lambs were maintained in isolation until the end of the experiment at 45 days of age. They were fed a commercial ewe milk replacer diet 2-4 times daily but introduced to irradiated lamb ration and autoclaved hay from 28 days of age.

Experimental gnotobiotic lambs

Gnotobiotic lambs were delivered by hysterectomy at 143 days gestation. Principals and controls were housed separately in pairs in plastic isolators. The lambs were fed 50-100 ml of 10% glucose within the first 2 hours of life, followed by 3 equally spaced feeds of glucose and milk over the remainder of the first

day. Thereafter the lambs were fed on milk 3 times daily until the end of the experiment at 21 days of age. Milk was sterilized by an ultra-high-temperature process and aseptically filled into aluminium foil lined cartons (Ford, Porter, Thompson, Toothill and Edwards-Webb, 1969). Every 3-4 days faecal swabs, placed in 2.5% Oxoid nutrient broth, were cultured as above to monitor any contamination of the isolators.

2.2.3. Lagomorphs

Lop-eared rabbits, 6-9 months of age, were used for production of hyperimmune antisera (Section 2.9.1).

2.3. MAINTENANCE OF *CRYPTOSPORIDIUM* AND PREPARATION OF OOCYSTS

The three isolates of *Cryptosporidium* used in this study were of bovine, equine and cervine origin, having been isolated from neonates of these species in which they were associated with diarrhoea. These isolates had the morphological and developmental characteristics of *Cryptosporidium parvum* (Tyzzer, 1912). The isolates were maintained by 3 to 4-monthly passage in conventional male calves or lambs (Section 2.2.2) housed in isolation facilities. Appendix 1 shows the passage history of each isolate. Only one isolate was passaged and processed at any one time. Between each passage, isolation facilities were thoroughly cleaned and animal isolators sprayed with aqueous 10% hydrogen peroxide, known to destroy *Cryptosporidium* oocysts (Blewett, 1988). Equipment used to attend the animals and process their faeces was cleaned and sterilized in a similar manner. Before the next passage isolators were again decontaminated for any *Cryptosporidium* oocysts by fumigation with 35% ammonia solution (sp. gr., 0.88) (Campbell *et al*, 1982).

Daily faecal examination prior to infection verified animals to be *Cryptosporidium* free. Examination for oocysts was done either

quantitatively in a haemocytometer using faeces diluted in aqueous 0.16% malachite green and 1% sodium dodecyl sulphate (SDS) or qualitatively by phenol auramine staining of faecal smears (Nichols and Thom, 1984). Calves or lambs at 5-7 days of age, which had been shown to be free of *Cryptosporidium* in this way, were dosed per os with 10^7 or 10^6 oocysts respectively.

A body harness with detachable faecal bag was fitted to each animal to allow collection of total daily faecal output. Faeces found to contain oocysts at a concentration of 10^6 per ml or greater were kept for subsequent extraction of oocysts. Animals were kept in isolators until oocysts were no longer detected in their faeces, usually 14-16 days post infection.

Oocysts were extracted and purified from faeces by sedimentation and differential centrifugation. An aqueous dilution of faeces (5%) was acidified with 2% sulphuric acid to a final pH of 5-6 which caused a large proportion of contaminating faecal debris to flocculate and sediment. Oocysts left suspended in the fluid phase were decanted, washed in tap water until the supernatant was clear, then resuspended in aqueous 1% SDS for 60 minutes at room temperature. Oocysts were then washed three times in tap water, resuspended in Hank's balanced salt solution (HBSS) (10^8 oocysts/ml) containing penicillin (Crystapen, Glaxo) and streptomycin (Evans) and stored at 4^0C. This final preparation was the oocyst stock from which experimental inocula were prepared.

2.4. EXCYSTATION TECHNIQUE

The method described here, which employs primary and secondary incubation phases, was used for all *in vivo* and *in vitro* experiments requiring *Cryptosporidium* sporozoites. Excystation occurs during the secondary incubation.

Procedure:

(A) Primary incubation.

The required number of oocysts from purified stocks were centrifuged and resuspended (up to 10^9 oocysts per ml) in 1% w/v trypsin (from beef pancreas, BDH Chemicals) in HBSS (pH between 3.5 and 4.0). This mixture was incubated for 1 hour at 37^0C in a water bath.

(B) Secondary incubation.

After the primary incubation oocysts were centrifuged, resuspended in excystation fluid to the same concentration and incubated at 37^0C for 30 minutes. Excystation fluid consisted of 0.05% w/v 7-deoxycholic acid, sodium salt (Sigma Chemicals) and 0.2% w/v sodium hydrogen carbonate in HBSS with a pH between 8.0 and 8.4.

The results of this excystation technique were quantified using methods similar to Fayer and Leek (1984) and Woodmansee (1986) but modified by Blewett (personal communication). After excystation the suspension was mixed by vortexing and 12µl placed on a microscope slide. A cover glass (22mm x 22mm) was placed on top and the edges sealed with mineral oil. The preparation was examined by phase contrast microscopy using a x40 objective lens and a total of 250 oocyst walls (shells), sporozoites and remaining oocysts counted on a differential cell counter. The excystation percentage, (number of shells x 100) / (number of shells + number of oocysts), and the sporozoite/shell ratio were calculated from this data. Sporozoite/shell ratios between 3 and 4 were considered normal.

2.5. INFECTION OF EXPERIMENTAL ANIMALS WITH *CRYPTOSPORIDIUM*

2.5.1. Lambs

Colostrum deprived and gnotobiotic lambs were infected per os at 5 days of age with a bacteria free inoculum containing 10^6 *Cryptosporidium* oocysts of the cervine isolate. Bacteria free oocysts were obtained by disinfection with 75% ethanol for 30 minutes (Tzipori *et al*, 1983) then pelleted (400xg, 5 min) and resuspended in sterile HBSS for dosing. The inoculum, consisting of 10^6 oocysts suspended in 2 ml of sterile HBSS was suckled by each lamb from an open 5ml syringe. Aerobic and anaerobic bacterial cultures were done, as before, on samples of inoculum intended for gnotobiotic lambs.

2.5.2. Rats and mice

Principals were infected at 4-7 days of age with defined doses of *Cryptosporidium* oocysts suspended in 100µl HBSS. Inoculations were performed without anaesthesia using a plastic stomach tube (30mm long, 0.8mm outer diameter) moulded over a 25 gauge needle. Bacteria free inocula (as above) were used with mice in experiments designed for collection of merozoites to be used as antigen. Purified inocula, diluted with HBSS to required concentration from oocyst stocks, were used in other experiments.

2.6. COLLECTION AND PREPARATION OF ANTEMORTEM SPECIMENS

2.6.1. Lamb faeces

Male lambs were fitted with faecal bag harnesses allowing collection of total daily faecal output. Faecal samples were obtained from the rectums of females. Examination for oocysts was done quantitatively in a haemocytometer using faeces diluted

in aqueous 0.16% malachite green containing 1% SDS and qualitatively by phenol auramine staining of faecal smears (Nichols and Thom, 1984).

Coproantibody was extracted from the faeces of principal and control male lambs with all procedures being carried out at 4^{0}C. Approximately 2 grams of faeces was ground up and diluted to a fluid consistency with a recorded volume of phosphate buffered saline (PBS, pH 7.1) containing 0.05% Tween 20 (Sigma Chemicals). The mixture was agitated on an orbital shaker for 60 minutes, centrifuged (10000xg, 5 min.) and the supernate, referred to hereafter as faecal extract, was stored at -20^{0}C. The final volume of the mixture was recorded after agitation and a dilution factor for each sample was calculated as follows : final volume / (final volume - volume of added PBS). Dilution factors ranged between 2 and 4. The lowest values were recorded for diarrhoeic faeces and the highest for preinoculation and post-diarrhoeic faeces.

2.6.2. Blood samples from lambs

Blood was collected from the jugular vein into evacuated bleeding tubes. Serum samples (off clots) were stored at -20^{0}C.

2.7. COLLECTION AND PREPARATION OF POSTMORTEM SPECIMENS

2.7.1. Lambs

Lambs were anaesthetized with 2ml "Saffan" (Glaxo) given intravenously, decapitated and hung by their hind limbs until bleeding was complete (approximately 3 minutes). The method of euthanasia allowed as complete an exsanguination as possible so that blood did not pool in abdominal viscera.

Small and large intestines were removed and dissected away from their mesenteries. The small intestine was separated at the ileocaecal junction, cut into 7 segments and each opened along the antimesenteric border. Each segment was approximately 2 metres long. Caecum and colon were separated and opened in a similar fashion. The caecal mucosa was cut into 3 strips (each approximately 15mm wide) from the proximal to distal end. The colon was approximately 2 metres long. Mucosal surfaces were usually free of faecal material as the animals were killed after an 18 hour fasting period. Any remaining faecal material was gently removed without disturbing the intestinal mucus layer.

Histology

Lengths of tissue (3.5cm long) from the middle and both ends of each of the 7 small intestinal segments were rolled, mucosa uppermost, around the circumference of 12mm diameter wooden dowels. This kept mucosal surfaces flat during fixation in Bouin's fluid (Drury and Wallington, 1967a), after which they were transferred to 70% ethyl alcohol. Tissues from the proximal end, middle and distal end of both caecum and colon were fixed in a similar manner. Fixed tissues were embedded in paraffin wax, sectioned 5μm thick and stained with either haematoxylin and eosin or Giemsa for light microscopy (Drury and Wallington, 1967b,c). Tissues from the duodenum, jejunum, terminal ileum (segments 1,4 and 7 respectively), caecum and colon were prepared for scanning electron microscopy according to the modified thiocarbohydrazide method of Malick and Wilson (1975). After critical point drying, specimens were mounted on stubs, sputter coated with a gold/palladium mixture with a Polaron E5100 sputter coater and viewed in a Jeol JSM-T300 scanning electron microscope. Mechanical agitation during processing was kept to a minimum to avoid dislodging surface mucus.

Intestinal mucus

After specimens had been taken for histology, each segment of intestine was immersed in 15ml of PBS for 2.5 hours at 4^0C. Caecal strips were immersed together. This change of temperature dislodged adherent mucus from the mucosal surface (Dobson, 1966). Surface mucus was then collected from small and large intestinal segments by drawing each of them through a measured slit in a broad rubber band. The apparatus used for this collection (see Figure 2.1) allowed the same gentle pressure to be applied to all intestinal segments. Mucus plus PBS was weighed for each segment of intestine and the mucus weight was calculated by subtraction. A dilution factor for each sample was calculated as follows : (weight of mucus + PBS, taken as 15g)/ weight of mucus.

2.7.2. Rats and Mice

Blood samples from rats

Blood was collected by exsanguination, either by cardiac puncture from rats (older than 21 days) anaesthetized with Halothane (May and Baker) or after cervical dislocation and decapitation of younger rats. Serum samples were stored at -20^0C.

Histology

Euthanasia was performed by cervical dislocation. In experiments designed to test sporozoite neutralization in rats, all of the small and large intestine was taken for histological examination. Small intestine and colon were opened along their antimesenteric border. Lengths of tissue (2.5cm long) were rolled, mucosa uppermost, around the circumference of 9mm diameter wooden dowels, to keep the mucosal surface flat, and fixed in Bouin's fluid (Drury and Wallington, 1967a). Circular

Figure 2.1. Apparatus used for collection of surface mucus from lamb small and large intestines. Intestine, shown schematically as the strip of paper(A), was slowly drawn through the slit in the rubber band(B). Mucus, skimmed from the mucosal surface, was collected into the cup(C) which contained phosphate buffered saline at 4^{0}C.


A
B
C

loops of tissue were sectioned transversely for light microscopy. The caecum was fixed as a whole and sectioned longitudinally.

In experiments designed to examine differences between rats and mice and in those examining multiple infections in rats, 1cm lengths of small intestine at 20% intervals were opened, mounted and fixed as above for rats. Specimens from proximal and distal colon were similarly prepared, while the stomach and caecum were fixed whole. Tissue from the small intestine was prepared for scanning electron microscopy. Processing of rat and mouse tissues for light microscopy and scanning electron microscopy was the same as that for the lamb. Tissues examined at the 0 and 20%, 40 and 60% and 80 and 100% intervals of small intestine were interpreted as duodenum, jejunum and ileum respectively.

Collection of mucus and merozoites

Surface mucus was collected in washings from the terminal ileum of sucking Swiss White mice. Ilea were processed in pairs. The last 20-30mm of unstretched ileum was dissected free of mesentery, removed, blotted free of blood and opened along the antimesenteric border. After faecal balls were removed, pairs of ilea were gently washed for 60 minutes at 20^{0}C in 2ml of PBS in a test tube mounted on an orbital cell mixer (Luckham CM100). After washing, the ilea were removed; one ml of the mucus containing fluid was centrifuged (200xg, 3 min) and the supernate stored at -20^{0}C. This centrifugation step pelleted any endogenous stages of *Cryptosporidium* together with dislodged epithelial cells and other insoluble material. A 0.1mm improved Neubauer haemocytometer, loaded with fluid from the remaining 1ml, was used to count merozoites and oocysts at daily intervals post infection. The remaining fluid was stored whole at -20^{0}C.

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2.8. INTERPRETATION OF INTESTINAL HISTOPATHOLOGY

Parameters which were observed to change during the course of infection included: 1. the severity of inflammation, 2. the population density of *Cryptosporidium* endogenous stages, 3. the percentage of villous enterocytes with large supranuclear vacuoles and 4. villous height and crypt depth. These parameters were either scored or measured in both principal and control animals.

2.8.1. Inflammation score

Inflammatory changes were scored for preweaned rats and mice. Severity was assessed subjectively by the intensity of cellular infiltrations into the mucosa as well as by changes in the intestinal epithelium (Table 2.1).

2.8.2. *Cryptosporidium* endogenous stage population density

This was determined by counting the number of *Cryptosporidium* endogenous stages in the microvillous border over 10 enterocyte nuclei. No attempt was made to distinguish between different endogenous stages (Table 2.1). A total population density score was calculated for each Lister rat by addition of individual organ population density scores from the duodenum, jejunum, ileum, caecum and colon.

2.8.3. Percentage of villous enterocytes with supranuclear vacuoles

This was determined by measuring, from the villous tip, what percentage of the villus was occupied by pinocytotically active vacuolated epithelial cells (Table 2.1).

Table 2.1. Interpretation and scoring of intestinal histopathology and the degree of Cryptosporidium infection

PARAMETER	SCORE: 0	1	2	3
Inflammation: lamina propria, submucosa	no change	Mild granulocytic infiltration of neutrophils(PMN) and eosinophils.	Moderate granulocytic infiltration: PMN and larger mononuclear cells (MON).	Intense cellular infiltrations: PMN and larger MON; possible microabscessation.
surface epithelium	no change	no change	Cuboidal /low columnar epithelium; partial villous atrophy.	Focal erosion of low cuboidal epithelium; exudation of PMN and MON into the lumen; partial villous atrophy.
Cryptosporidium endogenous stage population density: number of stages per 10 enterocyte nuclei.	not detected	<2	>2 and <10	>9
Percentage of villous epithelium with supranuclear vacuoles, as measured from the villous tip.	not detected	1 - 30	30 - 60	>60

2.8.4. Villous height and crypt depth

Measurements were made on villi and crypts in the small intestines of Lister rats. Only well-orientated villi and crypts were measured where the plane of section had passed through the villous tip and base of the adjacent crypt. Measurements were first made in millimeters on the screen of a projection microscope and then converted to micrometers using a conversion factor calculated from a stage micrometer viewed at the same magnification.

2.9. IMMUNOLOGICAL METHODS

2.9.1. Preparation of antisera against *Cryptosporidium*

Antisera against three *Cryptosporidium* isolates (bovine, equine and cervine) were raised in conventional Lop-eared rabbits (two animals per isolate). Antiserum against the bovine isolate, raised in a conventional lamb, was prepared by D.A. Blewett at the Moredun Research Institute. Antisera against the cervine isolate were raised in *Cryptosporidium* free inbred Lister rats. Animals were inoculated subcutaneously over the shoulder or flanks, intramuscularly in the mid-femoral region or intravenously into the jugular (lambs) or ear vein (rabbits).

The vaccination protocols for each species are shown in Tables 2.2a, 2.2b and 2.2c. Adjuvanted vaccines were made by emulsification of excysted oocysts with either complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA) and Tween 80 (Sigma Chemicals) using an homogenizer (MSE, England).

Table 2.2a. Vaccination schedule for raising antisera against Cryptosporidium in rabbits.

Elapsed time	No.of oocysts used[1] to produce antigen	Adjuvant	Inoculum dose and route per rabbit
Day 0	10^9	Freund's complete	1ml subcut., two sites
Day 21	10^9	Freund's incomplete	as above
Day 31	10^8	none	0.5ml I/V (ear vein)
Day 45	10^8	none	0.5ml I/V (ear vein)
Day 55	Rabbits anaesthetized with CO_2 and exsanguinated by cardiac puncture. Serum was stored at -20 ^{0}C.		

1. Oocysts were excysted (>80%). The products (a mixture of intact oocysts, oocyst walls and sporozoites) were inactivated by resuspending and freeze thawing in 200µl distilled water and then brought to 1ml with phosphate buffered saline (PBS), pH 7.1. Where indicated antigen was emulsified with either 1ml of Freund's complete or Freund's incomplete adjuvant and 2ml of 2% v/v Tween 80 in PBS before inoculation.

Table 2.2b. Vaccination schedule for raising antisera against Cryptosporidium in a conventional lamb.

Age(days)	Antigen	Adjuvant	Inoculum dose and route
5	10^6 oocysts[1]	none	orally
20	10^6 excysted oocysts[2]	Freund's complete	1ml subcut., 4 sites
41	10^6 excysted oocysts[2]	none	1ml I/V, jugular vein
51	Blood samples obtained from the jugular vein and stored at -20°C.		

1. Infected with oocysts suspended in 2ml Hank's balanced salt solution.
2. Oocysts were excysted (>80%). The products (a mixture of intact oocysts, oocyst walls and sporozoites) were inactivated by resuspending and freeze thawing in 200µl of distilled water and then brought to 1ml with phosphate buffered saline (PBS), pH 7.1. Where indicated antigen was emulsified with 1ml of Freund's complete adjuvant and 2ml of 2% v/v Tween 80 in PBS before inoculation.

Table 2.2c. Vaccination schedule for raising antisera against Cryptosporidium in Lister rats.

Age(days)	Antigen	Adjuvant	Inoculum dose and route per rat
15	10^6 oocysts[1]	none	orally
26	10^6 oocysts[1]	none	orally
48	10^6 excysted oocysts[2]	none	250µl I/M, 2 sites
55	10^6 excysted oocysts	none	250µl subcut., 2 sites
62	10^6 excysted oocysts	none	250µl I/M, 2 sites
70	Rats anaesthetized with Halothane (May and Baker) and exsanguinated by cardiac puncture.		

1. Infected with oocysts suspended in Hank's balanced salt solution.
2. Oocysts were excysted (>80%). The products (a mixture of intact oocysts, oocyst walls and sporozoites) were inactivated by resuspending and freeze thawing in 100µl of distilled water and then brought to 500µl with phosphate buffered saline, pH 7.1.

2.9.2. Specific antisera against immunoglobulins and whole sera

Antisheep sera

Antisera against sheep mu, alpha and gamma chains and whole sheep serum were raised in pigs and prepared by A.M. Dawson at the Moredun Research Institute. Immunoglobulin (Ig) G from antisera against sheep IgM, IgA and IgG was prepared by affinity chromatography using Protein A Sepharose CL4B (Pharmacia Ltd.). The IgG fractions were rendered monospecific, as determined by immunoelectrophoresis against whole sheep serum and double diffusion against purified preparations of sheep IgM, IgA and IgG, by passage through sheep $F(ab)_2$ and foetal lamb serum immobilized on Sepharose 4B (CN Br activated Sepharose 4B, Pharmacia Ltd.). The fraction binding to $F(ab)_2$ and eluting with 0.2M glycine HCl pH2.2 was retained as anti$F(ab)_2$ for subsequent conjugation.

IgG fractions with monospecific activity and an unabsorbed preparation of anti-sheep IgG with activity against heavy and light chains were conjugated to fluorescein isothiocyanate (FITC) by a modification of the method of Rinderknecht (1962). IgG was dialysed overnight against 0.25M sodium carbonate buffer (pH 9) containing 0.1M sodium chloride. The concentration of IgG was adjusted to 10-20 mg/ml and 0.5 mg of Celite containing 10% FITC (Sigma F1628) was added per mg of IgG (0.05 mg FITC/mg IgG) and the mixture stirred at room temperature for approximately 3 minutes. Celite was removed by centrifugation (1000xg, 3min) and the supernate passed through a column of Sephadex G25 (Pharmacia Ltd.) equilibrated with phosphate buffered saline pH 7.4. The labelled IgG was contained in the first coloured band eluting from the column.

Horseradish peroxidase (HRPO) was conjugated to the IgG containing monospecific antibody and to the anti$F(ab)_2$ antibody eluted from the $F(ab)_2$ Sepharose column by the method of Wilson and Nakane (1978).

All immunoglobulin specific antisera used in this study, both those produced at the Moredun Institute and those commercially available, are described in Table 2.3.

2.9.3. Detection of specific antibodies to *Cryptosporidium*

Indirect immunofluorescence assay (IFA)

Test antigen consisted of a mixture of sporozoites, shells and intact oocysts. These were prepared by excysting 10^7 *Cryptosporidium* oocysts (cervine isolate), which were subsequently spun down and resuspended in 300ul of HBSS. Sufficient antigen was spotted onto 15-well multitest slides (Flow Laboratories) to leave a wet film. The slides were air dried, wrapped in tissue paper, then in aluminium foil and stored at -20^0C. Test specimens were sera and extracts of faeces or intestinal mucus from infected and uninfected control animals. Test dilutions, rinsing and washing procedures were done using PBS. Doubling dilutions of test specimens were made starting from 1:10 for lamb sera, 1:5 for rat sera and neat extracts of faeces and mucus. End point titres were expressed as natural logarithms.

Before use, antigen slides were held at 4^0C overnight and allowed to reach room temperature before being fixed in acetone for 10 minutes. Test samples were spotted (10ul) onto antigen wells, incubated at room temperature for 40 minutes in a humid chamber, then rinsed. Slides were then washed (stirred) for 15 minutes, drained and gently blotted on filter paper (Whatman No.1). FITC labelled anti-globulin conjugates (Table 2.3), diluted to working concentration in PBS, were spotted (10ul) onto antigen wells. Slides were incubated, rinsed and washed as before. They were then examined on a Leitz Ortholux microscope with incident-light illumination, using a x50 water immersion objective lens.

Table 2.3. Immunoglobulin specific antisera used in this study

Antibody to	Raised in	Conjugation and working dilution	Source
Ovine:			
whole serum	pig	N[2]	Moredun Institute
IgM(H)[1]	pig	FITC(50)[3],HRPO(200)[4],N	" "
IgA(H)	pig	FITC(50), HRPO(50), N	" "
IgG(H)	pig	FITC(100), HRPO(200), N	" "
IgG(H+L)[1]	pig	FITC(200)	" "
$F(ab)_2$	pig	HRPO(200)	" "
Rabbit:			
immunoglobulin	pig	HRPO(400)	Dakopatts P217 (Denmark)
Rat:			
immunoglobulin	rabbit	FITC(100), HRPO(200)	Dakopatts F234, P162 (Denmark)

1. H: immunoglobulin heavy chain specific.
   H+L: immunoglobulin heavy and light chain specific.
2. N: unconjugated (used neat).
3. FITC: fluorescein isothiocyanate (reciprocal working dilution).
4. HRPO: horseradish peroxidase (reciprocal working dilution).

2.9.4. Column chromatography of sera and intestinal mucus extracts

This was carried out with Bio-Gel A-1.5m (Bio-Rad Laboratories) and Protein G Sepharose 4 fast flow (Pharmacia Ltd). These columns, the parameters of which are given at appropriate points in the text, were kindly prepared by Mr. A. M. Dawson (Pathology Department, Moredun Research Institute). Both columns were eluted at 4^{0}C and all effluents were monitored at 280nm with a Uvicord 2 (LKB). Fractions, collected by means of a fraction collector, were then concentrated and dialysed against PBS using Collodion Bags (Sartorius Instruments, SM132 OOE). Specific antibodies to *Cryptosporidium*, contained within fractions, were titred by IFA.

2.9.5. Agar gel diffusion

This was carried out according to the method of Ouchterlony (1949). Immunodiffusion was done with a variety of well patterns in 1% w/v Agarose (LKB) in Tris-barbiturate buffer (0.12M diethylbarbituric acid, 0.36M Tris, 0.005M calcium lactate, 0.01M sodium azide, pH 8.6), which had been poured in 12ml aliquots onto glass plates (84 x 94mm). After filling the wells, plates were placed in a humid chamber and left for diffusion at room temperature for 24 hours.

After diffusion the gel was washed from the plate in 0.1M sodium chloride and mounted on "Gelbond" film (LKB). After pressing and washing three times in 0.1M sodium chloride, the gel was dried to a thin film using hot air from a hair drier. The dried gel was stained for 5 minutes in 0.5% w/v Coomassie brilliant blue R-250 in ethanol/acetic acid/distilled water (9:2:9) and destained for 10 minutes. The destaining solution was the same as the staining solution but without Coomassie brilliant blue.

2.9.6. Immunoelectrophoresis

This was carried out according to the method of Grabar and Williams (1953). Tris-barbiturate buffer (as above) pH 8.6 was used in the tank. Twelve ml of agarose gel solution (as for agar gel diffusion) was poured onto glass plates (84 x 94mm). Antigen samples (10µl) were applied to 4.0mm diameter wells cut in the hardened agar and electrophoresis was carried out for 1 hour at 90 volts. After electrophoresis troughs were cut and antisera (100µl) applied before the plate was placed in a humid chamber overnight at room temperature. Pressing, drying and staining procedures were the same as those described for agar gel diffusion.

2.9.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Antigen preparation

Oocyst and merozoite antigens for SDS-PAGE were always prepared from the cervine isolate of *Cryptosporidium*.

Oocyst antigen

Oocysts were collected from the pooled faeces of either experimentally infected conventional or gnotobiotic male lambs and excysted (Section 2.3 and 2.4). The excystation products, consisting of unexcysted oocysts (<5%), sporozoites and shells, were centrifuged (10000xg, 60 sec) and the sediment retained. Sporozoites in the sediment were disrupted by 3 times freezing and thawing to 4^0C. The sediment was then resuspended in sample denaturing buffer (5% v/v 2-mercaptoethanol, 5% w/v SDS, 10% w/v sucrose and 0.002% w/v bromophenol blue in 63mM Tris-hydrochloric acid buffer pH 6.8), boiled for 3 minutes and then centrifuged (10000xg, 60 sec). The supernate was retained as oocyst antigen and stored at -20^0C.

For an estimation of their protein content oocysts were excysted and the products centrifuged to retain the sediment. Sporozoites in the sediment were disrupted by freezing and thawing as above. The sediment was then resuspended in distilled water and the mixture sonicated (5x, at 16μ amplitude for 60 sec with 60 sec rest between each sonication, in an MSE 150W sonicator) before centrifugation (10000xg, 60 sec) to retain the supernate. The protein content of the supernate was measured using the BCA Protein Assay Reagent (Pierce Chemical Company). Using this method it was determined that approximately 50μg of protein was derived from 10^7 excysted oocysts.

Merozoite antigen

Merozoites were collected in mucus washings from the terminal ileum of Swiss White mice infected at 7 days of age with a bacteria free inoculum containing 10^6 oocysts (cervine isolate). Mice were killed 3 days after inoculation and their ilea prepared as described in Section 2.7.2. Medium designed to minimize bacterial contamination and allow more rapid dispersion of mucus was prepared as follows: 2mM DL-dithiothreitol (Sigma Chemicals), 100 units/ml penicillin (Crystapen - Glaxo), 100μg/ml streptomycin sulphate (Evans) and 100mg/ml Terramycin soluble powder (Pfizer) in PBS. Ilea from 10 mice were placed in a test tube containing 10ml of this medium (freshly prepared) and gently agitated on an orbital cell mixer at 20^0C for 30 minutes. After mixing, the ilea were allowed to settle and a sample of the medium was loaded into a 0.1mm haemocytometer for estimation of merozoite numbers. The ilea were removed and the medium was centrifuged slowly (100xg, 3min) to separate merozoites (suspended) from much of the particulate debris and any contaminating oocysts. The supernate was centrifuged harder (2000xg, 10min, 4^0C) and the sediment , containing merozoites, was retained. Merozoite rich sediments were resuspended and pooled in 1.5ml of PBS, counted using a haemocytometer and

stored at -20^{0}C after being deposited by centrifugation (10000xg, 1min, 4^{0}C).

Ilea from age-matched uninfected control mice were prepared and treated in the same manner to those from infected mice. Sediments collected from the ilea of uninfected mice were stored at -20^{0}C and used as merozoite control antigen.

Merozoite rich sediment, collected from infected mice, and sediment from the same number of uninfected mice were resuspended in equal volumes of denaturing buffer and boiled as above. After centrifugation the supernates were saved as antigens for SDS-PAGE and stored at -20^{0}C.

Gel preparation

A discontinuous system was used, employing slab gels 0.75mm thick with a 12.5% resolving gel and a 3% stacking gel with the buffer system of Laemmli (1970). Resolving gel solution consisted of 12.5% w/v acrylamide, 0.35% w/v N,N^{1} -(1,2-dihydroxyethylene) bisacrylamide, 0.1% w/v SDS and 5% w/v sucrose in 0.38M Tris-hydrochloric acid buffer (pH 8.8). Polymerization was initiated by the addition of 80µl of 10% w/v ammonium persulphate and 12.5µl of N,N,N,N^{1} -tetramethylethylenediamine for each 40ml of resolving gel solution. Stacking gel solution consisted of 3% w/v acrylamide, 0.08% w/v N,N^{1}- (1,2-dihydroxyethylene) bisacrylamide, 0.1% w/v SDS in 0.125M Tris-hydrochloric acid buffer (pH 6.8). Polymerization was initiated in a similar way and the solution layered over that of the resolving gel. Gels were cast in a Bio-Rad casting apparatus between 160x180mm glass plates.

Electrophoresis

For immunoblotting experiments in which a number of different sera were to be reacted against the same antigen mixture gels were loaded in either of two ways:

(a) antigen was loaded into gels cast with a single large well and the subsequent immunoblots developed as a number of strips or

(b) antigen was loaded into wells cast by a 20 lane comb. Antigen lanes were separated between lanes carrying prestained molecular weight markers (Sigma SDS-7B). This method allowed better conservation of antigen and subsequent immunoblots were developed as strips cut out between lanes carrying transferred prestained molecular weight markers. Unless otherwise stated in the figure legend, all immunoblots were carried out using this method.

Gel electrophoresis was carried out using a reservoir buffer containing 0.025M Tris, 0.192M glycine and 0.1% w/v SDS (pH 8.3). Gels were run under constant current at 18mA for approximately 4.5 hours (until the marker dye front had migrated to within 1 cm of the end of the resolving gel).

Two sources of molecular weight markers were used:

Sigma Chemical Company Ltd., prestained molecular weight markers.

	molecular weight
human, alpha 2-macroglobulin	180,000
Escherichia coli, B-galactosidase	116,000
rabbit muscle, fructose-6-phosphate kinase	84,000
chicken muscle, pyruvate kinase	58,000

porcine heart, fumarase	48,500
rabbit muscle, lactic dehydrogenase	36,500
rabbit muscle, triosephosphate isomerase	26,600

BDH Ltd., Electran molecular weight markers.

	molecular weight
hen egg, ovotransferrin	76-78,000
bovine serum albumin	66,250
hen egg, ovalbumin	45,000
bovine chymotrypsinogen A	25,700
equine myoglobin	17,200
equine cytochrome c	12,300

The molecular weights marked on the figures in this thesis are given for the position of migration of the molecular weight markers themselves. Specific molecular weights given for antigens were calculated by reference to a standard curve, derived from the plot of the relative mobilities of the reference proteins against the $\log_{10}$ of their molecular weights (Shapiro, Vinuela and Maizel, 1967).

Staining of gels

Gels were stained by immersion and gentle agitation for 1 hour in a solution of trichloracetic acid, acetic acid, ethanol and distilled water (volume ratio, 1:7.5:50:70 respectively) containing 0.03% w/v Coomassie brilliant blue R250.

Destaining was in 2 stages, first, rapid destaining for 5 minutes by immersion in a solution of acetic acid, methanol and distilled water (volume ratio, 1:5:4 respectively), secondly, controlled destaining to final intensity by immersion in a solution of acetic acid, methanol and distilled water (volume ratio, 1:1:8 respectively).

2.9.8. Immunoblotting procedure

Electroblotting

Proteins were electrophoresed from the gels to nitrocellulose paper with a semi-dry electroblotting apparatus built at the Moredun Research Institute based on a design by Khyse-Anderson (1984). This involved the compression and electrophoresis of a filter paper/nitrocellulose/polyacrylamide gel/filter paper sandwich between graphite electrodes using a three buffer system. The filter paper used was Whatman 3mm chromatography paper. Two sheets of filter paper, soaked in a buffer containing 0.3M Tris in 20% v/v methanol at pH 10.4, were laid over the graphite surface of the anode. A further sheet of filter paper, soaked in a buffer containing 25mM Tris in 20% v/v methanol at pH 10.4, was placed on top of this. The nitrocellulose sheet, pre-wetted with distilled water, was placed on top and overlaid by the polyacrylamide gel. Care was taken to exclude air bubbles from under the gel. The sandwich was completed by overlaying two sheets of filter paper soaked in a buffer containing 25mM Tris and 40mM 6-amino-n-hexanoic acid in 20% v/v methanol at pH 9.4. This was covered by the graphite plate of the cathode. Electrophoresis was completed at room temperature by connecting the electroblotter to a power supply delivering a current of 0.8mA/cm^2 of the gel for 1 hour. Examination of the gel with Coomassie blue, before and after electrophoresis, showed that all major antigen bands had transferred using this method.

Immunological probing of blotted antigens

Following transfer, the nitrocellulose paper was trimmed and cut into strips. Blotted molecular weight standards and an adjacent antigen lane were trimmed from the edge and stained with a dilute solution of Coomassie blue. This was the Coomassie blue

stain for gels diluted 25 times in a solution of 30% v/v methanol and 5% v/v acetic acid. Destaining was done by immersion in a solution of 30% v/v methanol and 5% v/v acetic acid.

Nitrocellulose strips were agitated in wash buffer (1mM ethylenediamine tetra-acetic acid and 0.35M sodium chloride in PBS) containing 10% w/v ovalbumin (Sigma) for 18 hours at 4^0C. Sera or faecal extracts, diluted in wash buffer containing ovalbumin, were then agitated with the strips for 3 hours at room temperature. After three 10-minute washes, strips were agitated for one hour in the presence of excess HRPO labelled anti-globulin diluted in wash buffer with ovalbumin. After further washing, 3,3' diaminobenzidine (DAB) substrate was added and the colour reaction was stopped with water after approximately 3 minutes.

Substrate solution consisted of 0.1M Tris (pH 7.4) containing 0.04% w/v DAB, to which 40µl of 30% w/v hydrogen peroxide was added, just before use, for each 25 ml of solution used. Before substrate was added to washed nitrocellulose strips, a sample was used to test for a positive reaction with post incubation anti-globulin solution.

2.10. ANALYSIS OF MUCUS

2.10.1. Lambs

After collection into PBS, mucus gel from each segment of intestine (Section 2.7.1) was homogenized in a Potter S shear homogenizer (1500 rpm for 2 min at 4^0C) to solubilize mucus glycoprotein (Allen, Bell, Mantle and Pearson, 1982). The homogenate was centrifuged (10000xg, 15 min , 4^0C) to produce a crude soluble mucus extract in the supernate and a sediment of cell debris and other insoluble material. The supernate was stored at -20^0C until analysis.

After thawing and thorough vortex mixing, mucus extracts were analysed in 4 steps:

1. IFA (Section 2.9.3), to determine the titres of IgM, IgA and IgG which were specific for *Cryptosporidium* sporozoites.

2. Fractionation of selected samples by gel filtration (Section 2.9.4), using Bio-Gel A-1.5m (Bio-Rad), to separate the large molecular weight mucus glycoprotein (excluded) from the majority of lower molecular weight proteins. The method was similar to that used by Mantle and Allen (1981).

3. The distribution of IgM, IgA and IgG in these fractions was determined by agar gel diffusion using antisera specific for these immunoglobulins (Section 2.9.5).

Similarly, the distribution of some other proteins was determined by immunoelectrophoresis using antiserum raised against sheep serum (Section 2.9.6).

4. The concentration of mucus glycoprotein in the fractions was determined.

Hexoses, especially galactose and N-acetylgalactosamine, form one of the major groups of constituents of mucus glycoproteins (Marshall and Allen, 1977; Mantle and Allen, 1981). An estimate of hexose was therefore used as a measure of mucus glycoprotein. Results were expressed relative to galactose standards using the colourimetric method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). This involved hydrolysis with concentrated sulphuric acid, in the presence of a constant phenol concentration, producing a stable orange-yellow colour. Absorbance was measured at 490nm for hexoses and compared with freshly made standards of D(+) galactose (BDH Chemicals) at concentrations from 0-100µg/ml in distilled water.

This step-wise approach to mucus analysis was carried out because, in addition to mucus glycoproteins, gastrointestinal secretions contain serum proteins including antibodies and other substances in serum transudates (Horowitz, 1977).

2.10.2. Mice

Crude soluble and whole mucus samples were prepared from intestinal washings (Section 2.7.2) and stored at -20^{0}C until analysed as a single batch. The amount of mucus per sample was estimated by measurement of hexose (Bradbury, Black and Wyllie, 1980). Hexose analysis was performed by the phenol-sulphuric acid method (Dubois *et al*, 1956) using D-galactose as the standard.

2.11. THE USE OF *CRYPTOSPORIDIUM* SPOROZOITES IN *IN VITRO* AND *IN VIVO* EXPERIMENTS

2.11.1. *In vitro* assessment of the effects of serum and mucus on *Cryptosporidium* sporozoites

The effects, on sporozoites, of incubating them at 37^{0}C in dilutions of sera or mucus from immune and non-immune lambs were assessed on the basis of sporozoite/shell ratio and percentage sporozoites agglutinated.

Assessment of sporozoite lysis by measurement of sporozoite/shell ratio

Reference control values for this assessment were based on excystation of 10^{6} *Cryptosporidium* oocysts (cervine isolate) in 100µl of excystation fluid (Section 2.4). After excystation, the suspension was mixed by drawing up and gently expelling 50µl three times using a pipette. A cover glass (22mm x 22mm) was

placed over 12µl of the mixed suspension on a microscope slide and the edges were sealed with mineral oil. This preparation was examined by phase contrast microscopy using a x40 objective lens. The sporozoite/shell ratio and percentage excystation, calculated as previously described, were always at least 3 and 90% respectively. After centrifugation (10000xg, 10 sec) sporozoites, shells and remaining oocysts were resuspended in 100µl of fresh excystation fluid and incubated for a further 30 minutes at 37^{0}C. Sporozoite/shell ratios were calculated at 10, 20 and 30 minutes into this incubation and compared with that before resuspension in fresh excystation fluid (taken as time 0 minutes).

Reference values were compared with those of sporozoites incubated in dilutions of immune and non-immune sera or mucus under the same test conditions. The sporozoite/shell ratio for time 0 minutes was always made on the initial excystation mixture, before centrifugation and resuspension of sporozoites and shells in 100µl of diluted serum or mucus. Dilutions of sera and mucus extracts were made with fresh excystation fluid. Loss of sporozoites through lysis was reflected in a decreased sporozoite/shell ratio.

Unless otherwise stated, ratios at 0, 10, 20 and 30 minutes were made on three separate excystations and the mean values calculated. Sporozoites which appeared intact under phase contrast microscopy were included in the assessment while lysed, fragmented sporozoites were not.

Sporozoite agglutination in serum and mucus specimens

Reference control values for this assessment were measured, after incubation at 37^{0}C for 30 minutes, using the specimens prepared for calculation of control sporozoite/shell ratios described above. Agglutination was measured by counting 250 sporozoites into 3 categories, those existing singly, those in

pairs and those agglutinated in groups of 3 or more. Agglutination was calculated from the sum of the latter two categories divided by 250 and the result expressed as a percentage.

Reference values were compared with those of sporozoites incubated, under the same test conditions, in (a) 1:10 dilutions of heat-inactivated (56^0C, 30min) sera and (b) Bio-Gel A-1.5m fractions of colonic mucus, from immune and non-immune lambs. Dilutions were made with excystation fluid (Section 2.4). Agglutination values were the mean of measurements from 3 separate tests.

2.11.2. *In vivo* sporozoite infectivity

The following procedure was used to determine whether an infection could be established by inoculating an excystation mixture (Section 2.4) directly into the intestine of young rats.

Lister rats (5 days of age) were each inoculated per rectum with excystation mixture from 10^6 *Cryptosporidium* oocysts (cervine isolate). Sporozoites were inoculated, suspended with shells and remaining oocysts, in 100µl of excystation fluid. The excystation percentages for these inocula were at least 90%. The inoculum was delivered to the proximal colon by introducing 25mm of a 30mm length of plastic tubing (outer diameter 0.8mm) through the anus. The tubing was moulded over the end of a 25 gauge needle attached to a 100µl graduated syringe containing inoculum. Rats were killed in approximate half-litter groups at 3 or 5 days after inoculation. The entire small and large intestines were prepared for histological examination (as described in Section 2.7.2) from which the proportion of infected rats was determined.

Further groups of 5-day old Lister rats were similarly inoculated with excystation mixture from 10^5, 10^4 or 10^3

oocysts. These inocula were prepared by excysting 10^6 oocysts and making appropriate dilutions with excystation fluid. The inoculating volume was always maintained at 100µl so that the same surface area of gut was exposed regardless of dose. Rats were killed 3 or 5 days after infection and their intestines prepared as before.

Immediately after a rat had been inoculated, its rectum was blocked to prevent the escape of fluid. This was accomplished by introducing a glass rod (1cm long, 1.5mm diameter) sufficiently far into the rectum so that the end just protruded to facilitate later removal. The internal end was expanded by moulding over it a 0.5cm length of soft tubing (2.5mm diameter). These plugs were left in place for 1.5 hours during which time the pups were housed individually in open topped plastic cups. The plugs were then removed and the rats were returned to their dams.

2.11.3. *In vitro* sporozoite neutralization

Cryptosporidium sporozoites (cervine isolate) were incubated at 37^0C for 30 minutes in 1:10 dilutions of immune or non-immune, heat-inactivated (56^0C, 30 min), serum specimens. Sporozoites were derived from oocysts after excystation (Section 2.4). Although the excystation percentage was at least 90%, sporozoite preparations were contaminated by small numbers of intact oocysts. The infectivity of treated sporozoites and remaining oocysts was assessed by inoculating them, per rectum, into 5-day old Lister rats as described above. Each rat was infected at a dose rate equivalent to 10^6 or 10^4 oocysts. Excystation fluid was used to make dilutions of sporozoites and serum prior to incubation. The volume of inoculum for each rat was 100µl regardless of parasite numbers. Control rats were infected with the same doses but with sporozoites having been incubated only in excystation fluid at 37^0C for 30 minutes without exposure to serum.

2.12. STATISTICAL METHODS

Parametric data (measurements of hexose content, crude mucus weights and serum titres) were analysed by the Student's t-test (Snedecor and Cochran, 1967) on arithmetic values. Non-parametric data (histopathological scoring) were analysed by the Fisher Exact or Chi-Square tests as described by Siegel (1956). Values in the text are arithmetic means with either the standard deviation (SD) or standard error of the mean (SEM) indicated.

CHAPTER 3. Pathobiology of *Cryptosporidium* infection

EXPERIMENT 3.1. SELECTION OF A LABORATORY ANIMAL MODEL

Introduction

Laboratory rats, mice and guinea pigs have been reported to become infected when inoculated with *Cryptosporidium* oocysts but do not develop diarrhoea or any other obvious illness (Tzipori, Angus, Campbell and Gray, 1980a; Sherwood *et al*, 1982). In this experiment cryptosporidiosis in mice and rats was re-examined using 3 isolates of *Cryptosporidium*. The aim was to find a symptomatic small animal model with a distribution of infection similar to that in the lamb which was the main experimental domestic species used in this study. The model would then be used in comparative studies.

Materials and Methods

Three strains of mouse (randomly bred Swiss White, and inbred CBA and Porton WB) and three strains of rat (randomly bred Wistar and inbred F344 and Lister) were used. Each strain of rat and mouse was infected with three isolates of *Cryptosporidium*. Litters infected with different isolates were housed separately. The isolates, designated bovine, equine and cervine according to host species of origin, were administered orally (10^6 oocysts per animal) when litters were 4 days of age. Animals were killed in pairs and their intestines examined histologically on days 0, 2, 5, 8 and 11 after infection.

Histopathology was scored for severity of inflammation, *Cryptosporidium* endogenous stage population density and vacuolation of ileal enterocytes (Chapter 2.8). Age-matched uninfected control animals were examined in pairs in the same manner. Data was analysed firstly for differences between host species (mouse or rat), regardless of *Cryptosporidium* isolate

and secondly for differences between isolates (bovine, equine or cervine), regardless of host species. Table 3.1 shows the total number of samples examined and how they were collated on the basis of host species or infecting isolate. Significant differences were detected using the Chi square or Fisher Exact (2-tailed) tests where the total number of samples examined was greater or less than 40 respectively.

Results

Diarrhoea was observed only in infected rat pups. It was most severe in the Lister strain and evident for a variable period from 5-11 days after infection. The perianal region was stained with faeces and excoriated. On clinical examination pups were often observed to void a relatively large volume of fluid faeces.

Table 3.2 shows histopathological observations analysed for differences between host species. The duodenum and jejunum of the rat were infected more frequently than in the mouse ($p<0.001$). The mouse ileum showed a more severe infection, both in terms of population density ($p<0.01$) and accompanying inflammation ($p<0.001$) than the rat. This coincided with the more rapid replacement of vacuolated epithelium in the mouse ileum ($p<0.001$ at 5 days post infection). Similarly, the rat caecum more often carried a heavier infection ($p<0.001$) and showed more severe inflammation ($p<0.001$) compared to the mouse. There were no significant differences between mouse and rat for colonic infection. Cryptosporidial stages were never detected in the stomachs of infected mice or rats.

Table 3.3 shows histopathological observations analysed for differences between *Cryptosporidium* isolates. There were no significant differences detected between the isolates.

Table 3.1. Collation of data from histopathological observations on cryptosporidiosis based on host species or infecting isolate.

Intestinal site examined for:	(A) Days examined after infection	(B) No. mice(rats) examined on each day	(C) Strains of mice (rats) infected with each isolate	(D) No. of isolates	Total number of samples examined based on: Host species mice(rats) Infected (ABCD)*	Control (ABC)	Each isolate (mice and rats) Infected (ABC)	Control (ABC)
1. Population density score								
duodenum	2,5,8,11	2(2)	3(3)	3	72(72)	24(24)	48	48
jejunum	2,5,8,11	2(2)	3(3)	3	72(72)	24(24)	48	48
ileum	2,5,8,11	2(2)	3(3)	3	72(72)	24(24)	48	48
caecum	2,5,8,11	2(2)	3(3)	3	72(72)	24(24)	48	48
colon	2,5,8,11	2(2)	3(3)	3	72(72)	24(24)	48	48
2. Inflammation score.								
Same sites as those examined for population density score.								
3. Enterocyte vacuolation score.								
ileum	2	2(2)	3(3)	3	18(18)	6(6)	12	12
ileum	5	2(2)	3(3)	3	18(18)	6(6)	12	12

* That is: AxBxCxD

Table 3.2. Differences between rats and mice in the distribution of *Cryptosporidium* infection, severity of inflammation and loss of vacuolated ileal enterocytes.

1. Population density score:	Intestinal sites examined:	Infected Mice	Rats	
>1	duodenum	4[1]	43[1]	***[2]
>1	jejunum	3	46	***
>1	ileum	39	19	**
>2	caecum	29	54	***
>2	colon	33	27	NS
2. Inflammation score:				
>1	duodenum	0	0	NS
>1	jejunum	3	2	NS
>1	ileum	37	10	***
>2	caecum	15	35	***
>2	colon	25	17	NS
3. Enterocyte vacuolation score:				
0 (day 2)	ileum	0	0	NS
0 (day 5)	ileum	18	7	***

1. A total of 72 samples from each organ were scored for population density and inflammation, whereas 18 specimens of ileum were scored on each day for enterocyte vacuolation. Uninfected controls always had infection and inflammation scores of zero and vacuolation scores of 3.
2. Differences in the number of observations made between mice and rats were tested for significance using the Chi square test for population density and inflammation scores and the Fisher Exact test for enterocyte vacuolation scores.
NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 3.3. Differences between *Cryptosporidium* isolates based on the distribution of infection, severity of inflammation and loss of vacuolated ileal enterocytes in rats and mice.

		Isolates			
1. Population density score:	Intestinal sites examined:	bovine	equine	cervine	
⩾1	duodenum	14[1]	19[1]	14[1]	NS[2]
⩾1	jejunum	14	20	15	NS
⩾2	ileum	15	17	26	NS
⩾2	caecum	27	27	29	NS
⩾2	colon	18	18	24	NS
2. Inflammation score:					
⩾1	duodenum	0	0	0	NS
⩾1	jejunum	0	3	2	NS
⩾1	ileum	12	18	17	NS
⩾2	caecum	16	17	17	NS
⩾2	colon	10	13	19	NS
3. Enterocyte vacuolation score:					
0 (day 2)	ileum	0	0	0	NS
0 (day 5)	ileum	8	9	8	NS

1. A total of 48 samples from each organ were scored for population density and inflammation, whereas 12 specimens of ileum were scored on each day for enterocyte vacuolation. Uninfected controls always had infection and inflammation scores of zero and vacuolation scores of 3.
2. Differences in the number of observations made between isolates were tested for significance using the Chi square test for population density and inflammation scores and the Fisher Exact test for enterocyte vacuolation scores.
NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Control animals remained clinically normal and histological examinations never revealed inflammatory lesions or *Cryptosporidium* in their intestines. Vacuolated epithelium remained prominent in the ilea of control animals over the period of observation.

Discussion

The results indicated that significant differences in observations depended on host species and not on *Cryptosporidium* isolate. This finding may have been peculiar to the rodent strains and isolates studied. By using field isolates of *Cryptosporidium* in checkerboard infections, the rat emerged as the best laboratory animal candidate from which to define a suitable model. Like cryptosporidiosis in lambs (Angus *et al*, 1982b; Snodgrass *et al*, 1984) the infection in rat pups has been shown to result in diarrhoea and to be distributed along the entire small and large intestine. In mice, the ileum harboured the majority of endogenous stages in the small intestine and infections were asymptomatic.

The Lister rat was selected as a suitable laboratory animal host because infection was symptomatic, the distribution of infection was similar to that in the lamb and, by being an inbred strain, studies on immunity requiring adoptive cell-transfer could be facilitated. The cervine isolate of *Cryptosporidium* was chosen for the model because abundant purified stocks were available at a time when subsequent experiments were to commence.

EXPERIMENT 3.2. HISTOPATHOLOGICAL STUDIES ON NEONATAL, PREWEANING AND POSTWEANING LISTER RATS.

Introduction

Lambs, like other domestic species, have shown an age-related susceptibility to clinical cryptosporidiosis. Lambs infected at 10 days of age developed clinical disease, infection at 20 days resulted in little or no diarrhoea and 30-day old lambs became infected but remained healthy (Tzipori *et al*, 1981c). Similar age susceptibility to infection has been shown in immunocompetent laboratory mice but even as neonates they were clinically healthy (Sherwood *et al*, 1982).

The aim of this experiment was to examine age-related susceptibility with a histopathological study of changes occurring in the Lister rat over the course of single and multiple infection with *Cryptosporidium* (cervine isolate).

Experimental design

Litters of Lister rat pups were allocated to either control or to one of three infected groups:

1. Rats infected once, at 4 days of age, and killed 2, 5, 8, 11 or 14 days after infection.

2. Rats infected up to three times, at 4, 15 and 26 days of age, and killed on days 2, 5, 8 or 11 after the first infection and on days 5 or 11 after both of the subsequent infections.

3. Rats infected twice, at 15 and 26 days of age, and killed 2, 5, 8 or 11 days after the first infection and 5 or 11 days after the second infection.

Blood samples were taken from control and principal rats at the necropsy times indicated above. They were used for serological and immunoblot studies described in Chapters 4 and 5.

The dose on all occasions was 10^6 oocysts/rat. The intestines were fixed, prepared for histology and scored for inflammation, *Cryptosporidium* endogenous stage population density and enterocyte vacuolation as previously described (Chapter 2.7 and 2.8). A total infection score was calculated for each rat by addition of individual organ population density scores from the duodenum, jejunum, ileum, caecum and colon. For group 1 infected rats, villous height and crypt depth measurements were made (Chapter 2.8) and mesenteric lymph nodes were examined histologically.

Results

Clinical Signs

Rats infected at 4 days of age developed diarrhoea which was evident for a variable period from 5-11 days after infection. Rats did not show evidence of diarrhoea after 15 days of age, regardless of the infection regime.

Cryptosporidium endogenous stage population density

The changes in endogenous stage population density which occurred during the course of single and multiple infections with *Cryptosporidium* are shown in Figure 3.1. The greatest proliferation of endogenous stages occurred after a primary infection at 4 days of age. Following a single infection at 4 days (group 1) maximum population density was reached at 9 and 12 days of age in the small and large intestine respectively. The number of endogenous stages on infected epithelium fell below histologically detectable levels by 15, 15 and 18 days of age in duodenum, jejunum and ileum respectively. However,

Figure 3.1. Changes in the endogenous stage population density score during the course of single and multiple infections with *Cryptosporidium*. These changes are shown as individual organ population density scores in 3.1a and as total population density scores in 3.1b. Groups of Lister rats were infected once(▲, group 1), twice(●, group 3) and three times(■, group 2) with 10^6 oocysts (cervine isolate) at the ages indicated(↓). Each point represents the mean calculated from 3 rats after histopathological examination of the duodenum, jejunum, ileum, caecum and colon.

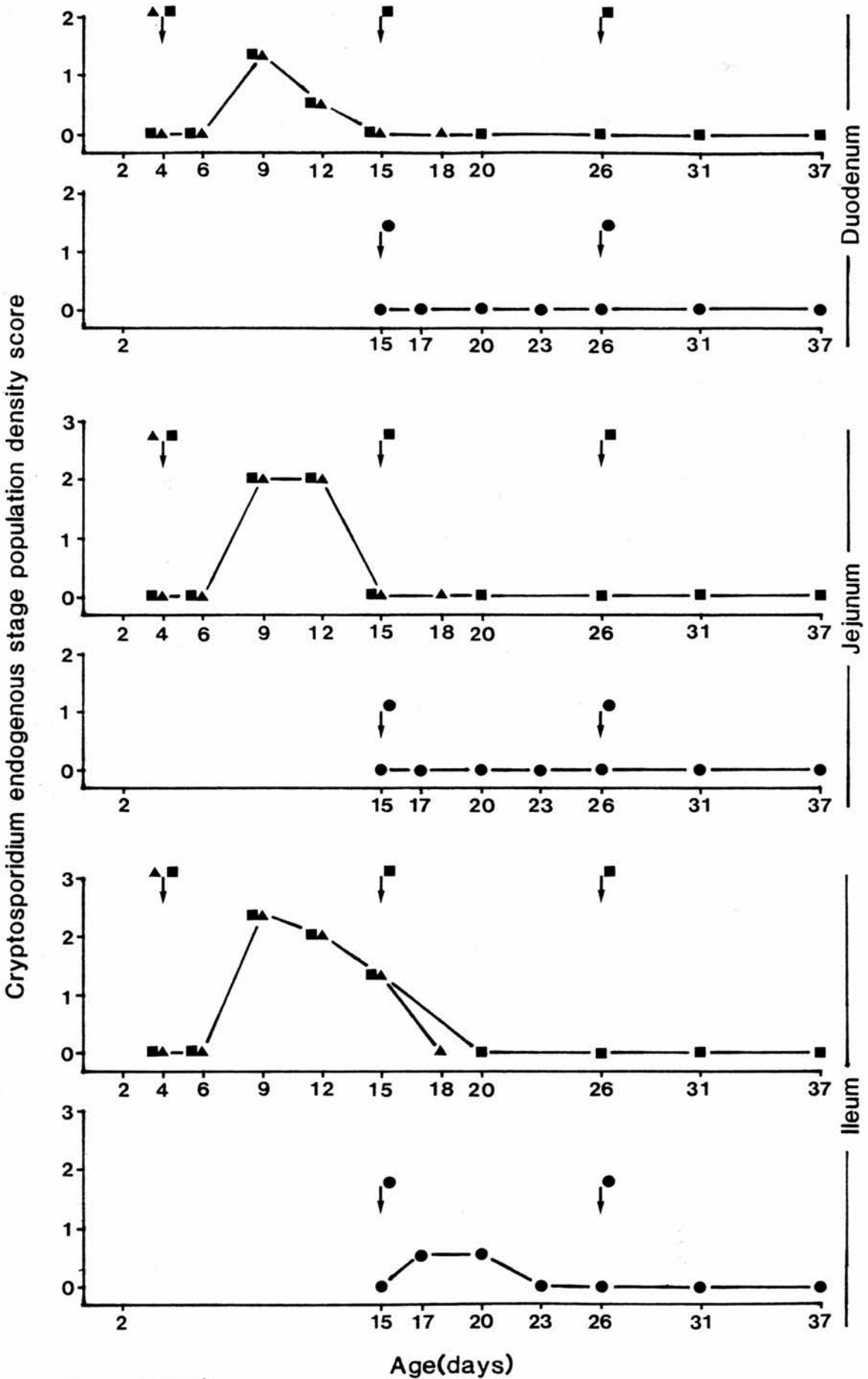

Cryptosporidium endogenous stage population density score
Duodenum
Jejunum
Ileum
Age(days)

Figure 3.1(a)

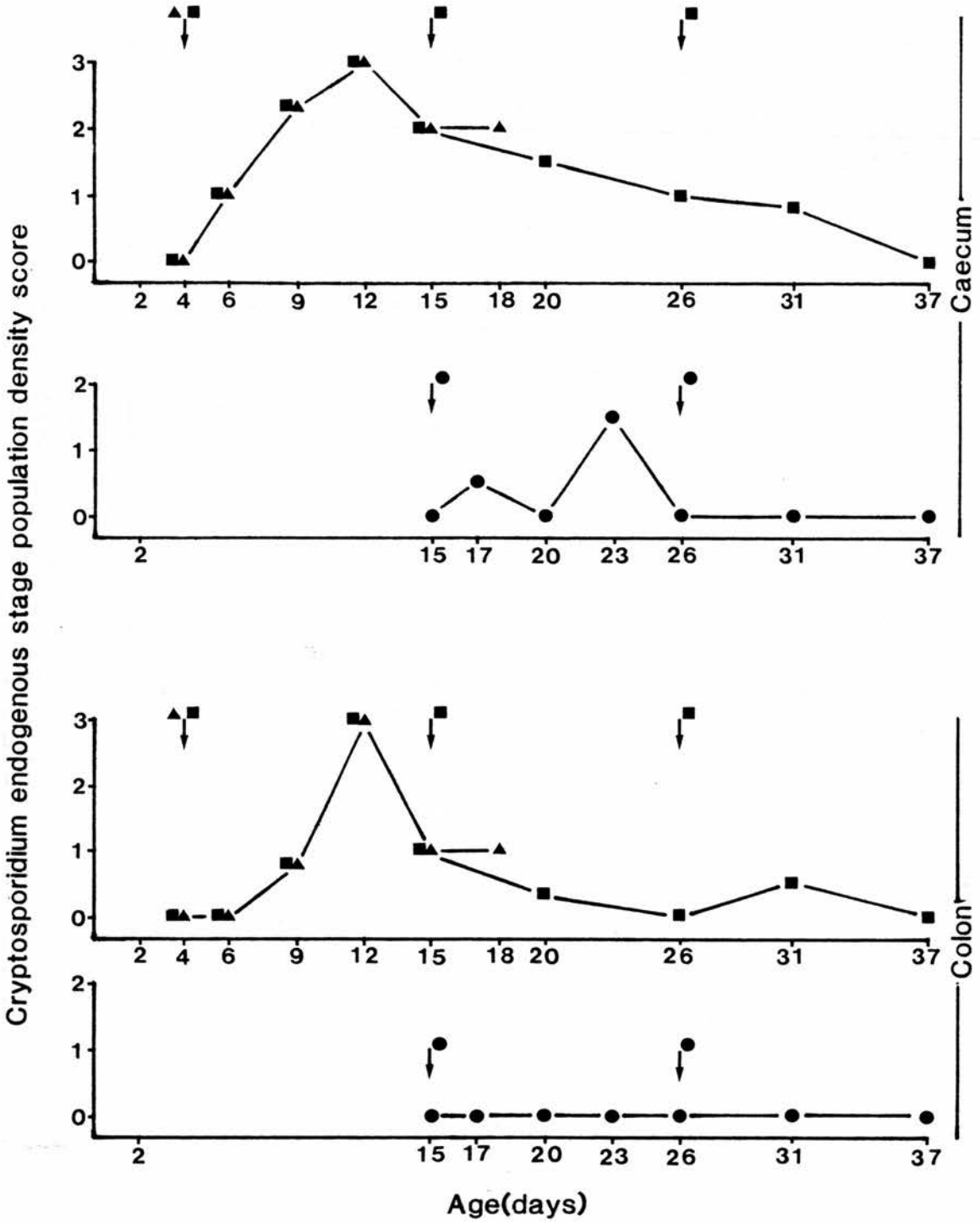


Figure 3.1(a) cont.

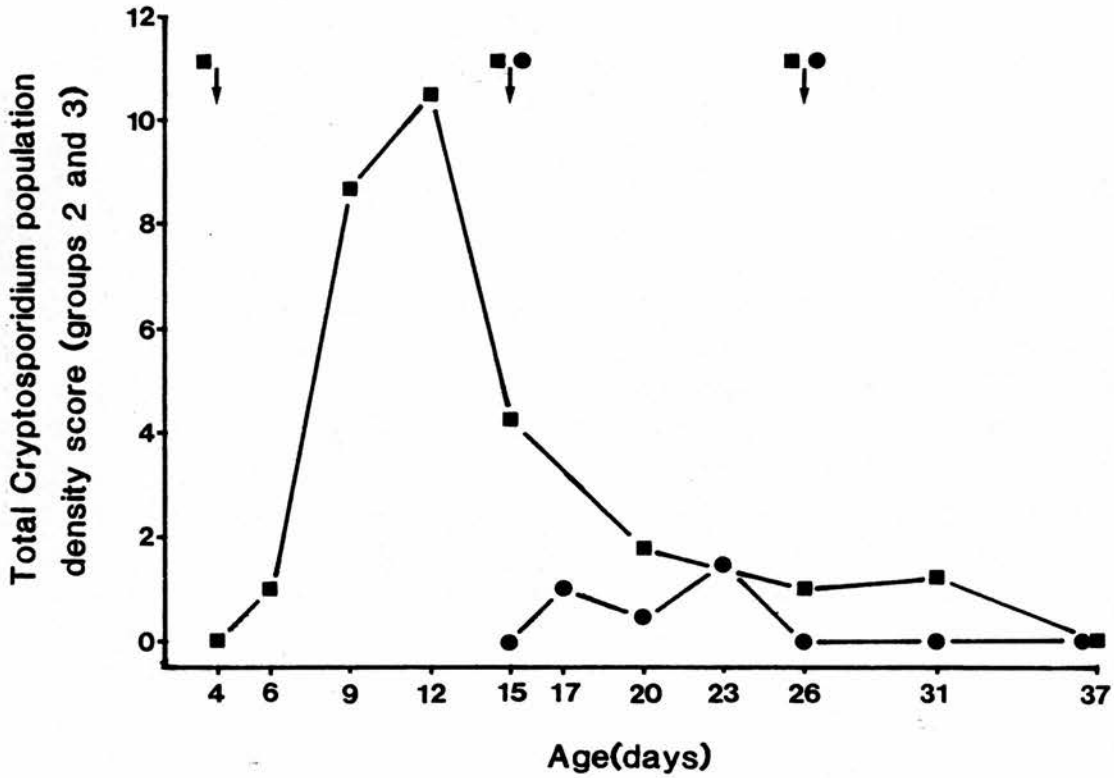

Total Cryptosporidium population density score (groups 2 and 3)
12
10
8
6
4
2
0
4
6
9
12
15
17
20
23
26
31
37
Age(days)

Figure 3.1(b)

moderate numbers of endogenous stages were still detected on caecal and colonic epithelium at 18 days of age. After the second and third infections, at 15 and 26 days of age (group 2), endogenous stages were not detected in the small intestine. However, they were detected in caecum or colon at 20, 26 and 31 days of age but not in animals examined at 37 days of age.

Following primary infection at 15 days (group 3) endogenous stages were detected up to 20 and 23 days of age in ileum and caecum respectively. A second infection at 26 days of age did not result in endogenous stages being detected in any gut segment.

Endogenous stages were never detected in control animals which remained serologically negative throughout the experiment (Chapter 4).

Enterocyte vacuolation

Enterocytes with large supranuclear vacuoles were most obvious in the ileum. In control rats these vacuolated cells occupied at least 60% of the upper villous length until 18 days of age. They were restricted to the villous tips in animals examined at 20 days and not detected in control animals 26 days of age. After this only the normal degenerative vacuolation was observed in small numbers of enterocytes sloughing at the villous extrusion zone.

Following a single infection at 4 days of age (group 1), supranuclear vacuolated enterocytes in the ileum were completely replaced by non-vacuolated columnar cells by 8 days post infection (Figure 3.2 a-d). Vacuolated enterocytes were again evident on ileal villi at 15 days of age but none were detected in animals examined at 22 days of age. Similarly, with animals infected a second and third time (group 2), vacuolated cells were not detected on ileal villi of those examined at 22 days of age or older.

Figure 3.2a-d. Histopathological changes in the ileum after infection of Lister rats with 10^6 *Cryptosporidium* oocysts at 4 days of age.

3.2a. Ileum from an uninfected control rat at 4 days of age.

3.2b,c,d. Ilea from rats examined at 2, 5 and 8 days after infection respectively.

Vacuolated ileal enterocytes, which occupied at least 60% of the upper villous length at 0 and 2 days after infection, were restricted to the top of the villus at 5 days and not detected in animals examined 8 days after infection. Numerous *Cryptosporidium* endogenous stages (arrows) were attached to the brush borders of enterocytes. At 8 days after infection, crypts were elongated and swollen atrophic villi were lined by a cuboidal to low columnar epithelium. All sections were stained with haematoxylin and eosin. Magnification factors are shown below each figure.

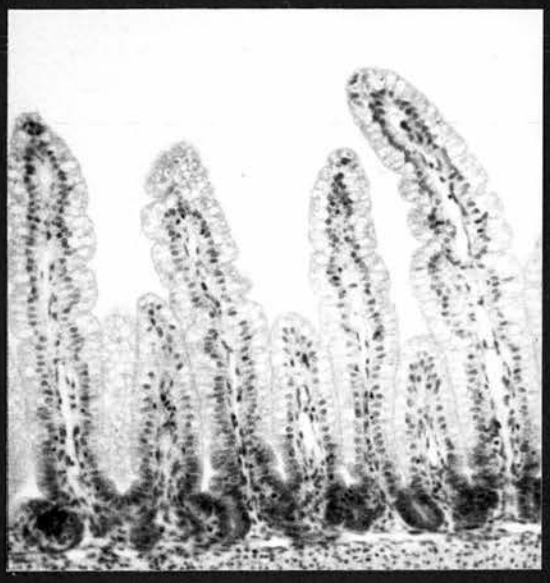


Figure 3.2a x400

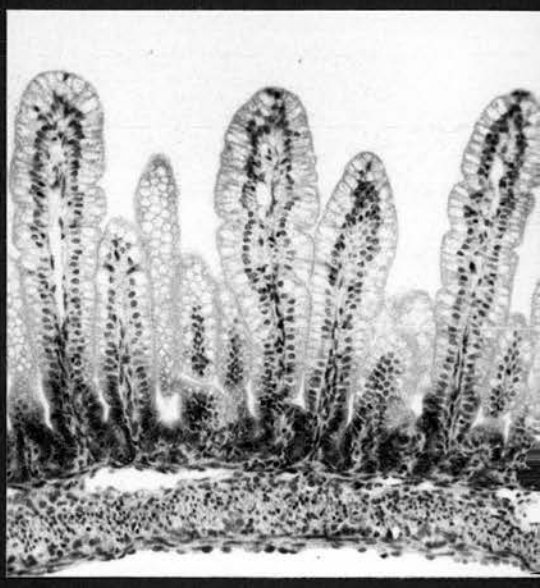


Figure 3.2b x400

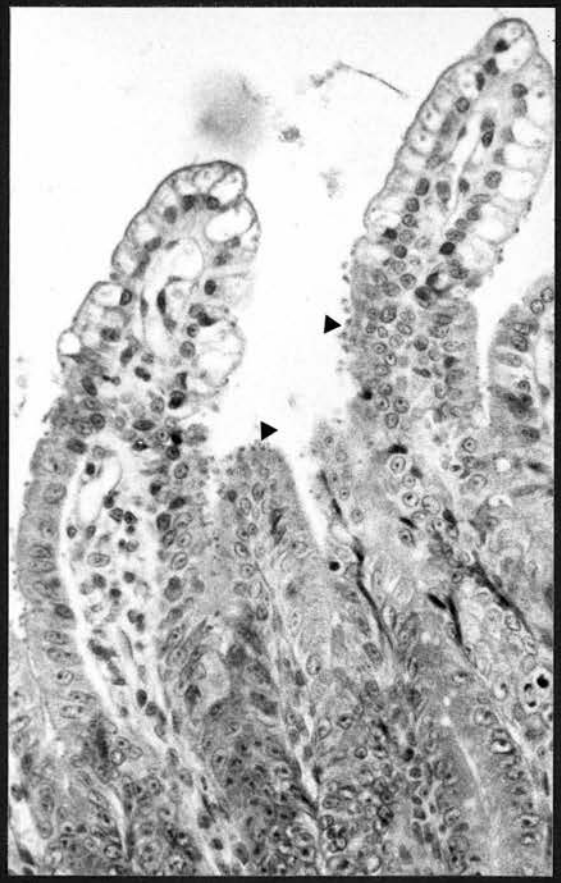


Figure 3.2c x1000

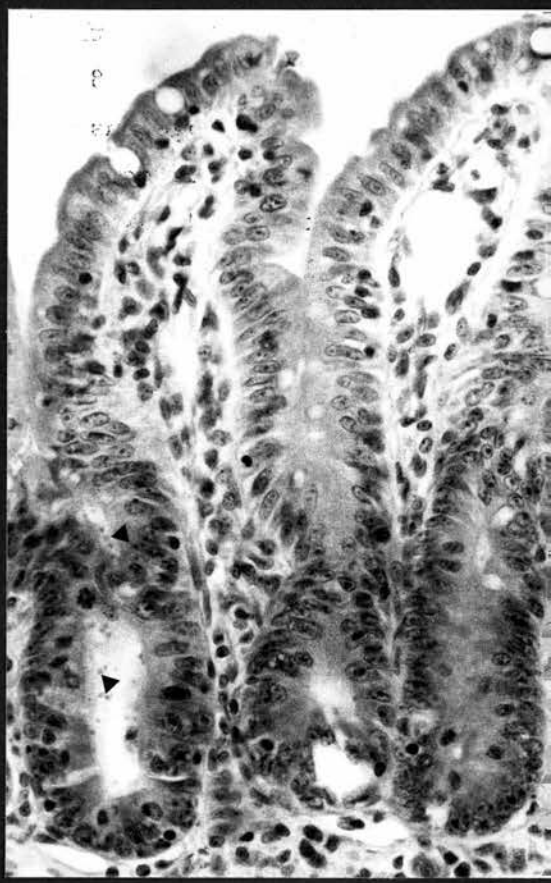


Figure 3.2d x1000

Following primary infection at 15 days of age (group 3) vacuolated ileal enterocytes were progressively restricted to the top of the villus and not detected at all in animals examined at 20 days of age or older.

Villous height and crypt depth

The changes in these parameters following a single infection at 4 days of age are shown in Figure 3.3. Villous atrophy was most evident in jejunum on days 5 and 8 and in ileum on day 8 after infection (Figures 3.2 and 3.4). Mean villous length for infected rats was less than half that of controls for jejunum on day 5 and ileum on day 8 after infection. Crypts in affected sites were elongated and often dilated. By 14 days after infection there were no apparent differences in villous-crypt architecture between control and infected rats.

Inflammatory response

This was most pronounced following single or multiple infections from 4 days of age. Inflammation in the small intestine was mild and not evident beyond 18 days of age. The epithelium covering atrophic villi was basophilic and of a cuboidal to low columnar type. Villous lamina propria and epithelium were infiltrated with small numbers of mononuclear inflammatory cells and neutrophils. Endogenous stages were seen attached to enterocytes at all levels of intestinal villi and in dilated crypts.

Moderate numbers of endogenous stages were attached to the epithelium of the bile duct as it passed through the wall of the duodenum. This was an incidental finding in a group 1 rat, at 31 days of age, 5 days after its second infection. The inflammatory response was as mild as that which accompanied endogenous stages in the small intestine of younger rats.

Figure 3.3. Lengths of villi and depths of crypts in the small intestine of Lister rats 2, 5, 8, 11 and 14 days post infection (pi) with 10^6 *Cryptosporidium* oocysts (cervine isolate). Age-matched controls were not infected. Bars represent the means (±SD) of 14-20 well orientated villi and crypts of two rats.

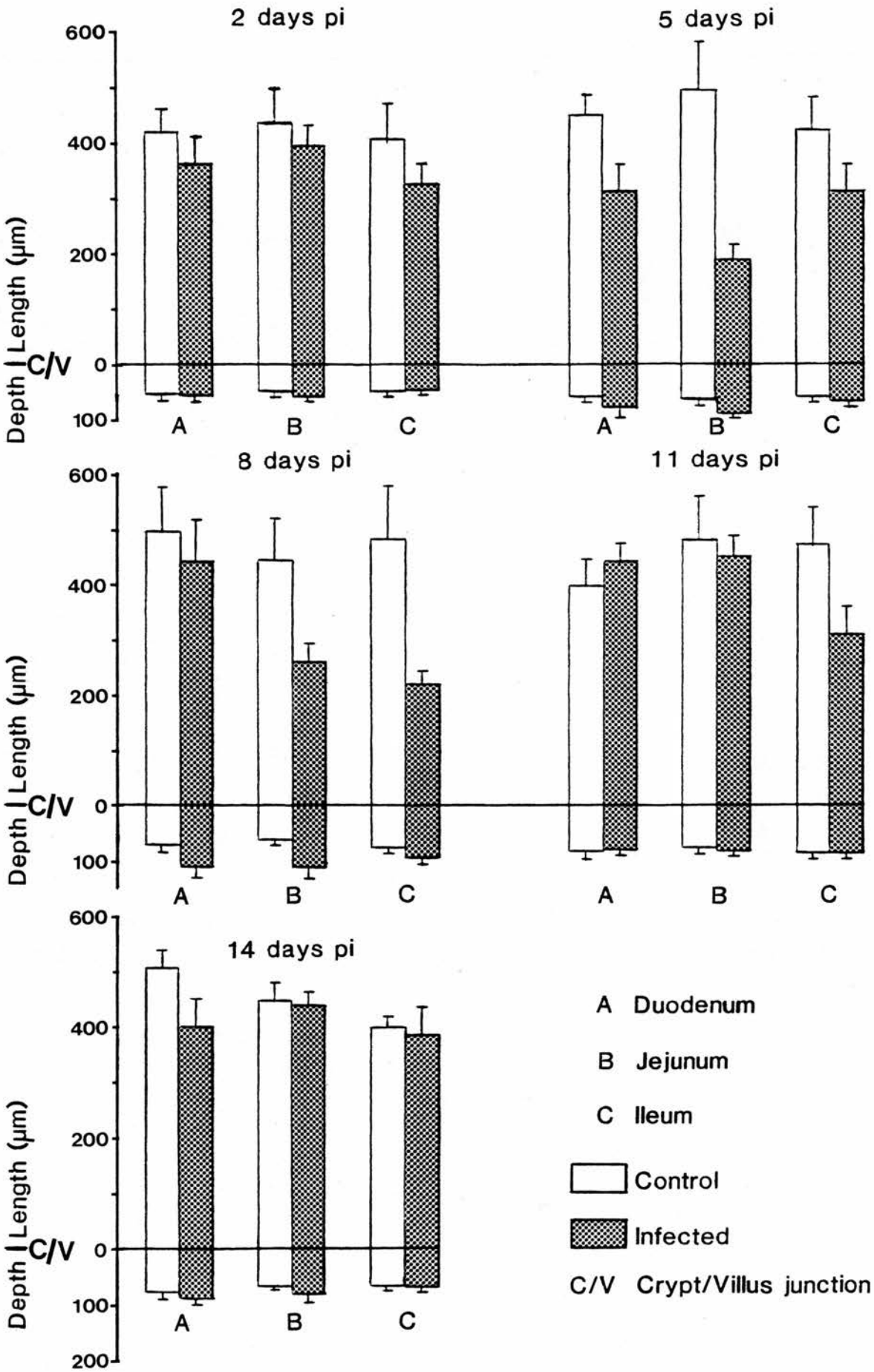

2 days pi
5 days pi
8 days pi
11 days pi
14 days pi
Depth | Length (µm)
600
400
200
0
100
200
C/V
A
B
C
A Duodenum
B Jejunum
C Ileum
Control
Infected
C/V Crypt/Villus junction

Figure 3.4a,b. Histopathological changes in the jejunum after infection of Lister rats with 10^6 *Cryptosporidium* oocysts at 4 days of age.

3.4a. Jejunum from an uninfected control rat at 9 days of age.

3.4b. Jejunum from a rat examined 5 days after infection.

In comparison to age-matched controls, infected jejunal mucosa showed swollen atrophic villi lined by cuboidal to low columnar epithelium and elongated crypts. Increased numbers of mononuclear inflammatory cells were infiltrating the lamina propria of atrophic villi. *Cryptosporidium* endogenous stages (arrows) were attached to enterocyte brush borders. Both sections were stained with haematoxylin and eosin. Magnification factors are shown below each figure.

Figure 3.4a x400

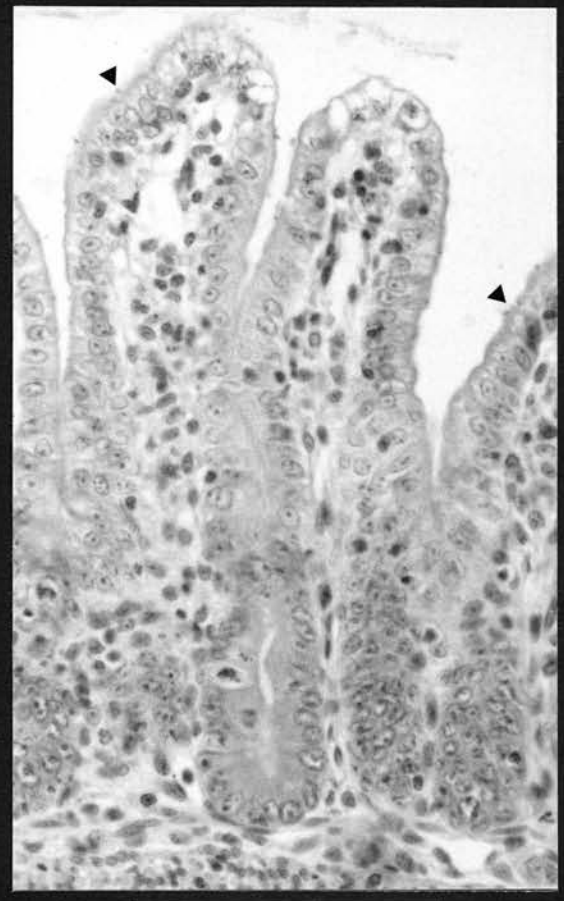


Figure 3.4b x1000

Figure 3.5a,b. Histopathological changes in the caecum after infection of Lister rats with 10^6 *Cryptosporidium* oocysts at 4 days of age.

3.5a. Caecum from an uninfected control rat at 12 days of age.

3.5b. Caecum from a rat examined 8 days after infection.

In comparison to age-matched controls, infected caecal mucosa showed disorganisation and degeneration of gland epithelium. This was associated with numerous attached *Cryptosporidium* endogenous stages (arrows) and infiltration of the lamina propria by large mononuclear inflammatory cells and neutrophils. Both sections were stained with haematoxylin and eosin. Magnification factors are shown below each figure.

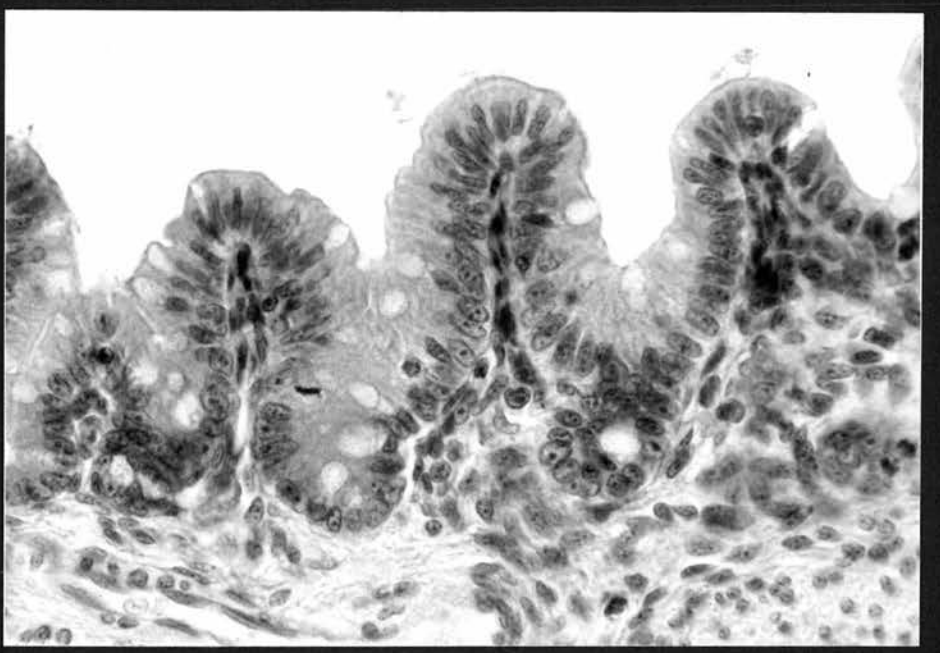


Figure 3.5a x1600

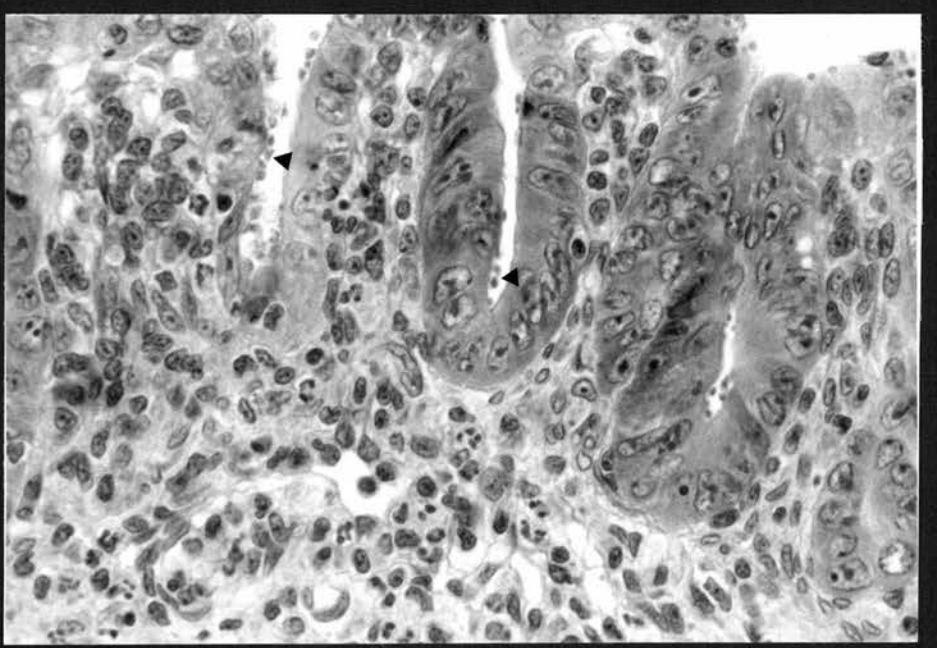


Figure 3.5b x1600

Inflammation was more severe in the large intestine, especially in the caecum, persisting in association with endogenous stages until 31 days of age. Large numbers of endogenous stages were seen attached to enterocytes, both on the surface and in crypts. Shrunken degenerating enterocytes with pyknotic nuclei were scattered within the epithelium. Focal erosion of superficial epithelium was occasionally seen in the caecum. The lamina propria was infiltrated by large numbers of mononuclear cells, including macrophages and lymphoid cells, and smaller numbers of neutrophils. Many crypts contained degenerating neutrophils (Figure 3.5a,b).

In the mesenteric lymph node, early primary lymphoid follicles were evident as condensations of cortical lymphoid tissue from 9-12 days of age in both control and infected rat pups. Early secondary follicles, with pale staining germinal centres, were seen from 15-18 days of age in mesenteric lymph nodes of infected but not control rats.

Following primary infection at 15 days of age, inflammation was detected only in the caecum, 8 days after infection, in association with endogenous stages. The response was mild and did not persist after secondary infection at 26 days of age.

Discussion

This experiment defined, in histological terms, a symptomatic model of cryptosporidiosis in the inbred Lister strain of laboratory rat which was used in later immunological studies. Diarrhoea was observed only in sucking rats infected at 4 days of age. Infections close to and after weaning (21 days of age) did not induce clinical signs, an observation which paralleled the published age susceptibility of young lambs (Tzipori *et al*, 1981c).

The entire length of intestine was not examined histologically and small numbers of endogenous stages may have remained undetected in sections. However, the results indicated that, after infection at 4 days of age, *Cryptosporidium* endogenous stages proliferated for a longer period in the large intestine compared to the small intestine. The single infection at 4 days of age was not followed through until endogenous stages were undetected in the large intestine. Hence, it is uncertain that the second and third infections in group 2 rats were responsible for prolonging infection in the large intestine.

Observations on rats infected for the first time at 15 days of age suggested that the ileum and caecum were the only portions of intestine favouring development of sufficient endogenous stages as to be histologically detectable. Unlike the infection in younger rats, endogenous stages were not detected in the digestively active proximal small intestine, a finding which may explain the absence of clinical signs in this age group.

Pinocytotically active villous epithelium, with large supranuclear vacuoles, is a normal feature of the ileum in preweaned rats. Over the weaning period, between 18 and 22 days of age, this epithelium is replaced by non-vacuolated columnar cells (Clarke and Hardy, 1969). In the present experiment vacuolated ileal enterocytes were finally replaced by 20-22 days of age regardless of the infection regime. However, rats infected at 4 days of age showed a premature but transient loss of supranuclear vacuolated enterocytes 8 days after infection, coinciding with severe villous atrophy in the ileum.

Mean villous length for infected animals was less than half that for controls for jejunum on day 5 and ileum on day 8 after infection. Even if the epithelial replacement rate remained the same in infected animals, this suggested that the migration time for cells along the villus would be at least halved. Reduced migration time in areas of severe villous atrophy may result in

undifferentiated trophozoite stages being lost into the gut lumen when enterocytes are extruded at the villous tip.

In conclusion, the results of this experiment indicated that in the Lister rat model:

1. diarrhoea was a feature of the disease in those animals infected at 4 days of age. Its duration coincided with peak parasite numbers and damage to the small and large intestinal mucosa.

2. after infection at 15 days endogenous stages were detected only in ileum and caecum. Diarrhoea was not observed in these animals.

3. after infection at 4 days the large intestine was the last site in which the numbers of endogenous stages fell to levels below that detectable by thorough histological examination.

4. reduced enterocyte migration time, in areas of severe villous atrophy, may limit the parasite's reproductive potential if undifferentiated trophozoites are sloughed with host cells at the villous tip.

EXPERIMENT 3.3. *IN VIVO* AND *IN VITRO* INVESTIGATIONS OF EXCYSTATION

Introduction

The results of Experiment 3.1 and the work of others (Current and Reese, 1986) showed that in murine cryptosporidiosis, the majority of endogenous stages in the small intestine were harboured in the ileum. This could be due to enterocytes of the proximal small intestine being relatively resistant to cryptosporidial infection or because excystation was localized in the distal small intestine. The aim of the present experiment was to investigate *in vivo* excystation in the mouse. The results were used to try to optimise conditions for *in vitro* excystation as a means of providing sporozoites for neutralization experiments in Chapter 6.

Experimental design

Gastrointestinal pH changes

Thirteen 7-day old Swiss White mice were each given two 50µl oral doses of a pH indicator dye 20 minutes apart. All mice were necropsied 30 minutes after the last dose. The gastrointestinal tract was removed, dissected free of mesentery, laid out and photographed. The indicator dyes used were BDH Chemical products and their pH ranges are shown in Figure 3.6. The dyes were used at a concentration of 1% w/v in distilled water. After dosing, mice were returned to their dams and suckled normally.

In vivo excystation

Eighteen Swiss White mice were infected orally with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 7 days of age and killed in groups of three at 1/4, 1, 2, 3, 4 and 5 hours after infection. At necropsy the small intestine was severed at the

ileocaecal junction, dissected free of mesentery, laid out and divided into 4 equal parts. Each segment was flushed vigorously with 1ml of 10% buffered neutral formalin. The number of sporozoites and oocysts recovered from each segment was counted using a 0.1mm haemocytometer and a x40 objective lens with phase contrast microscopy.

Two mice were infected orally with 10^8 oocysts. The first was killed 2 hours after infection and the second at 4 hours. At necropsy, the small intestines were removed, divided into 8 equal segments and tissue samples taken from each for histopathological and scanning electron microscopical examination.

In vitro excystation

Oocysts (cervine isolate) were excysted *in vitro* using the two step incubation procedure described in Chapter 2.4 but with the reagents tested under different concentration and pH conditions. The various conditions under which *in vitro* excystation was attempted are shown in Table 3.5. Oocysts, derived from a batch of purified stock (Chapter 2.3), were pelleted and resuspended in reagent solutions at a concentration of approximately $5x10^7$ per ml. Excystation percentages and sporozoite/shell ratios were calculated at the end of the first incubation and at 10, 20, and 40 minutes after commencement of the second incubation period.

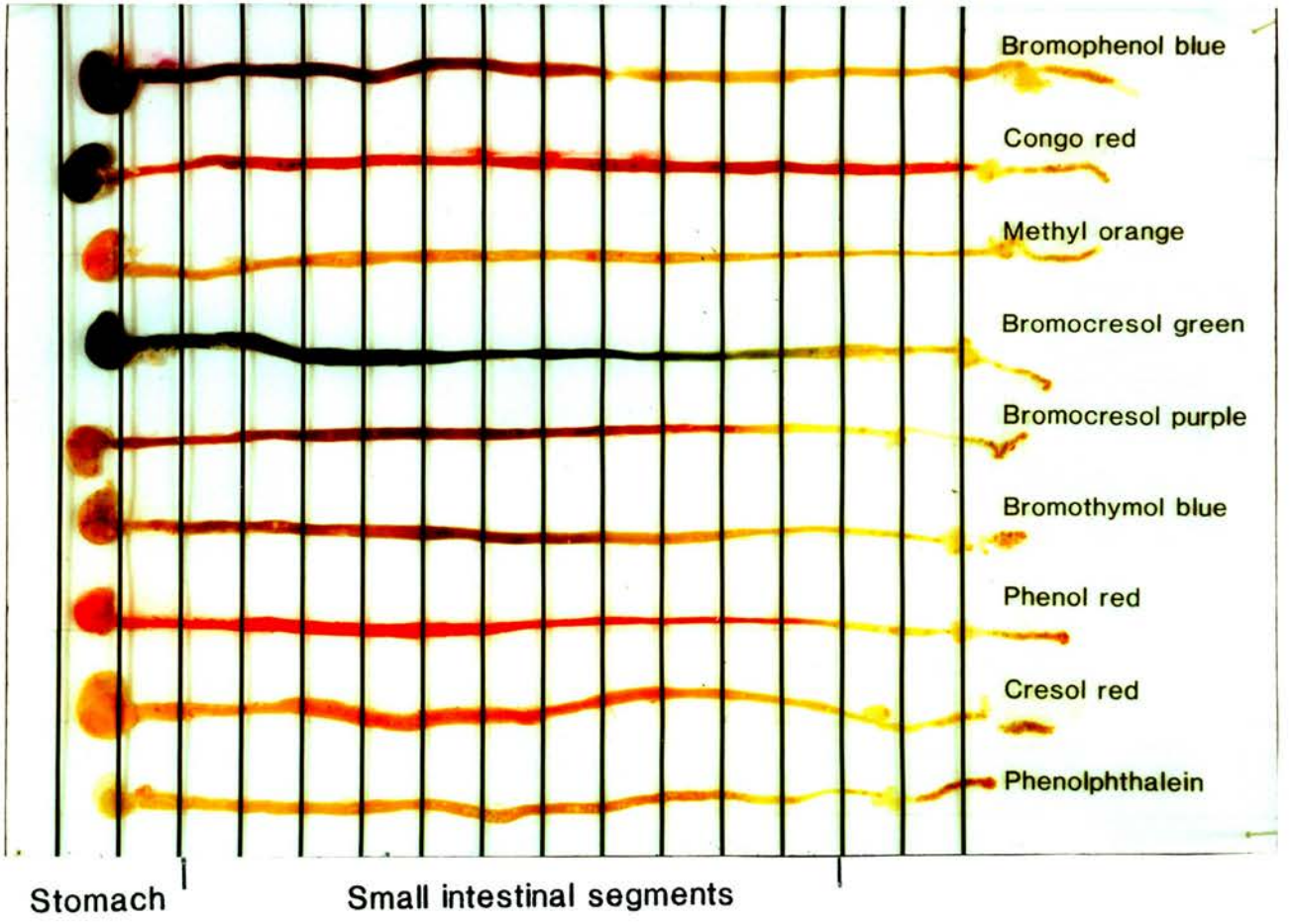
Results

Gastrointestinal pH changes

The colour of indicator dyes in the gastrointestinal tract of 9 mice and interpretation of them is given in Figure 3.6. The stomach contents by this method had a pH of approximately 5. Small intestinal pH appeared to lie between 7.6 (bromothymol

Figure 3.6. Changes in pH along the gastrointestinal tract of 7-day old Swiss White mice as shown by oral administration of selected indicator dyes. Dyes were used at a concentration of 1% w/v in distilled water. Mice were each given two 50µl doses of indicator dye 20 minutes apart and necropsied 30 minutes after the last dose. The indicator dyes used and their pH ranges were as follows:

Indicator	pH ranges
1. Bromophenol blue	yellow, 3.0----4.6, blue
2. Congo red	blue, 3.0----5.0, red
3. Methyl orange	red, 3.1----4.4, yellow
4. Bromocresol green	yellow, 3.8----5.4, blue
5. Bromocresol purple	yellow, 5.2----6.8, purple
6. Bromothymol blue	yellow, 6.0----7.6, blue
7. Phenol red	yellow, 6.8----8.4, red
8. Cresol red	yellow, 7.2----8.8, red
9. Phenolphthalein	colourl, 8.4---10.0, red



Indicator	pH indicated: Stomach	pH indicated: Small intestine
Bromophenol blue	≥4·6	≥4·6
Congo red	≥5·0	≥5·0
Methyl orange	≥4·4	≥4·4
Bromocresol green	≥5·4	≥5·4
Bromocresol purple	≤5·2	≥6·8
Bromothymol blue	≤6·0	≥7·6
Phenol red	≤6·8	≥8·4
Cresol red	≤7·2	7·2–8·8
Phenolphthalein	≤8·4	≤8·4

blue) and 8.4 (phenol red). Cresol red started to change colour at a point 50% along the small intestine indicating a pH between 7.2 and 8.8. These results were confirmed when bromocresol green, bromocresol purple, phenol red and cresol red were repeated in further mice so that colour changes could be better appreciated by opening the intestinal lumen at points along its length rather than interpreting colour through the gut wall.

In vivo excystation

Figure 3.7 shows the time course and distribution of oocysts and sporozoites after infection. Approximately 73% of recovered oocysts were from the first and second intestinal segments while 99% of recovered sporozoites were from the third and fourth intestinal segments (Table 3.4). The one and two hour intestinal flushes together, accounted for 79% and 97% of oocysts and sporozoites recovered from each mouse respectively. Based on an ideal of 4 sporozoites per oocyst, then by addition of oocyst count to 25% of the sporozoite count, it was estimated that 40% of the infective dose was recovered.

Table 3.4. The percentages of oocysts and sporozoites collected from each segment of mouse intestine over the 5 hour post infection period.

Intestinal segment	1	2	3	4
Oocysts (%)	48	25	14	13
Sporozoites (%)	0.3	0.2	6.5	93

Trophozoites were seen histologically only at 4 hours post infection, predominantly in the distal half of the small intestine. Scanning electron microscopy revealed numerous sporozoites associated with surface mucus in the distal small intestine (Figure 3.8a,b). Surface mucus with or without sporozoites was not a feature of the proximal small intestine.

Figure 3.7. Distribution of *Cryptosporidium* oocysts and sporozoites collected from mouse small intestine at 1/4, 1, 2, 3, 4 and 5 hours after infection with 10^6 oocysts. Each bar represents the mean of counts obtained from 3 mice.

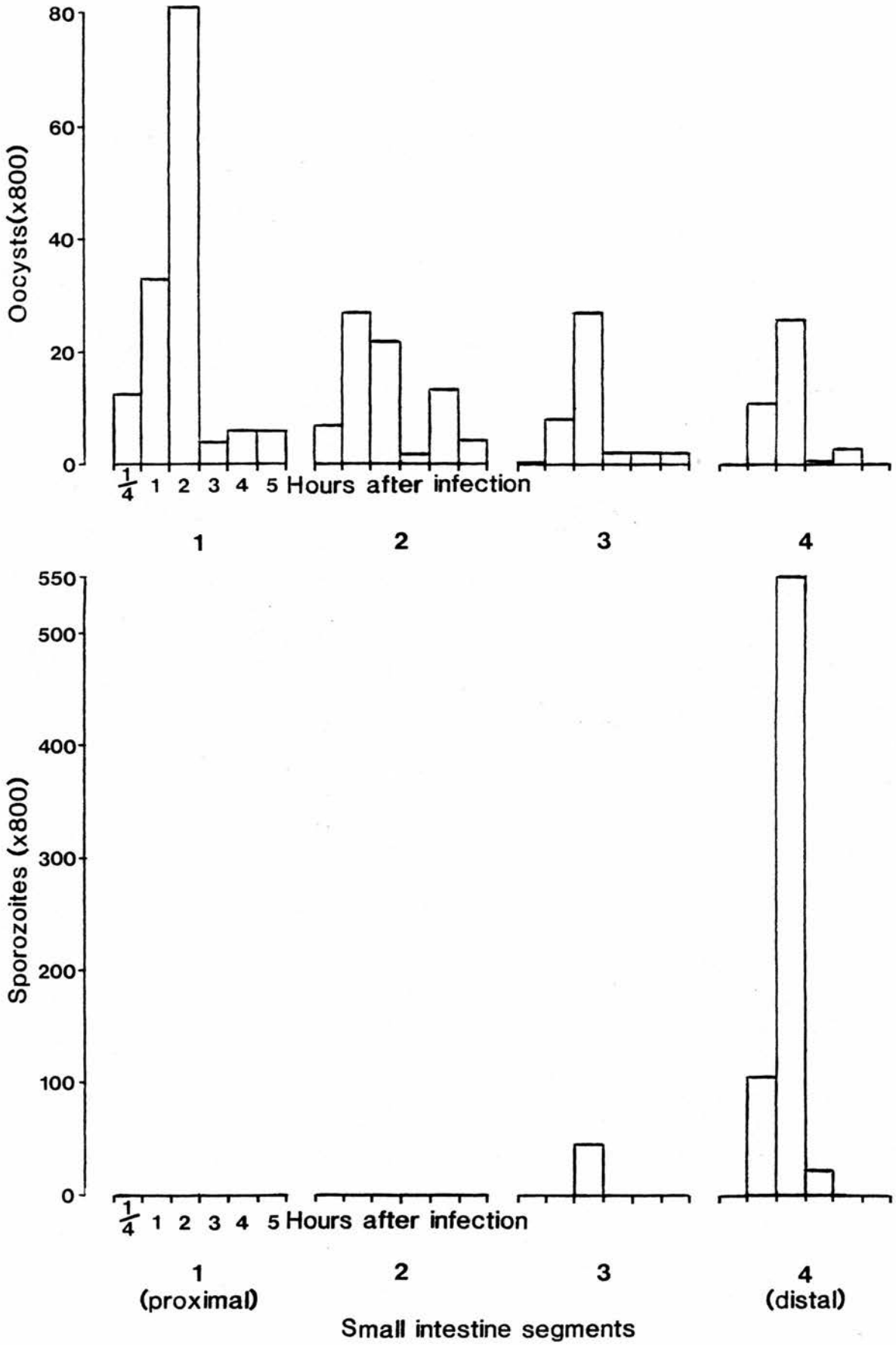

Oocysts(x800)
80
60
40
20
0
¼ 1 2 3 4 5 Hours after infection
1
2
3
4
Sporozoites (x800)
550
500
400
300
200
100
0
¼ 1 2 3 4 5 Hours after infection
1
(proximal)
2
3
4
(distal)
Small intestine segments

Figure 3.8a,b. Scanning electron micrographs of mouse ileum 4 hours after infection with 10^8 *Cryptosporidium* oocysts. Numerous sporozoites were found associated with mucus on the lateral surfaces (3.8a) and tips (3.8b) of villi. Magnification factors are shown at the bottom of each figure.

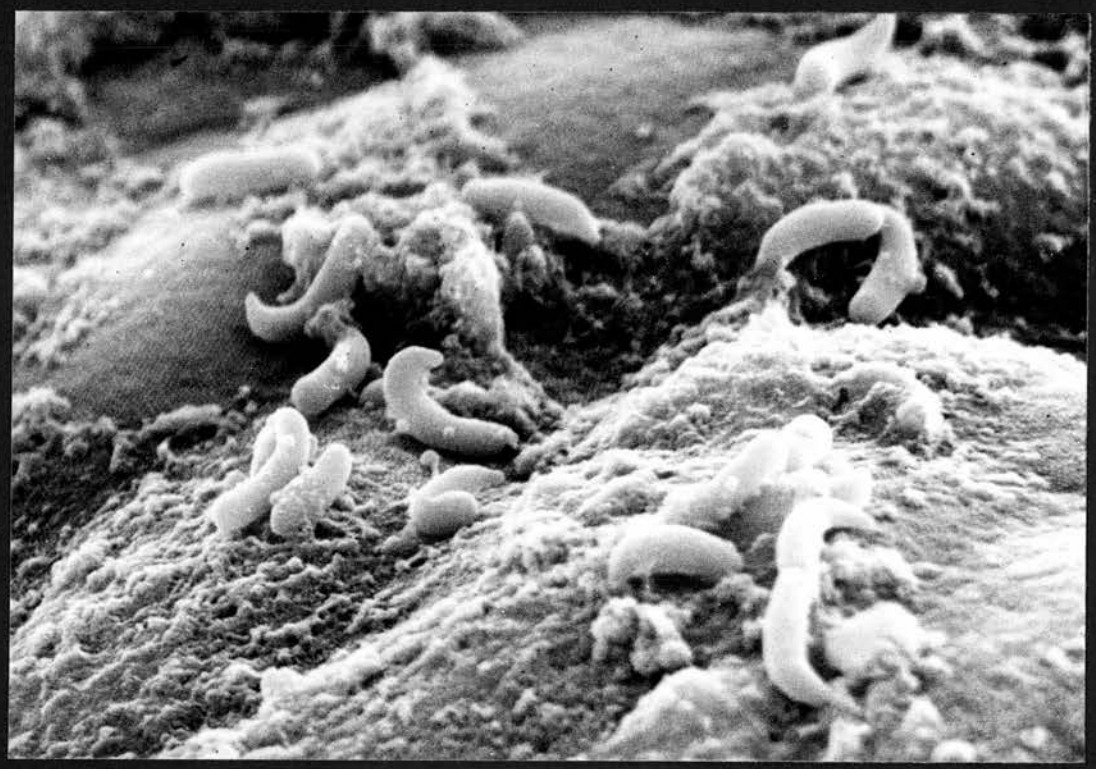


Figure 3.8a x4500

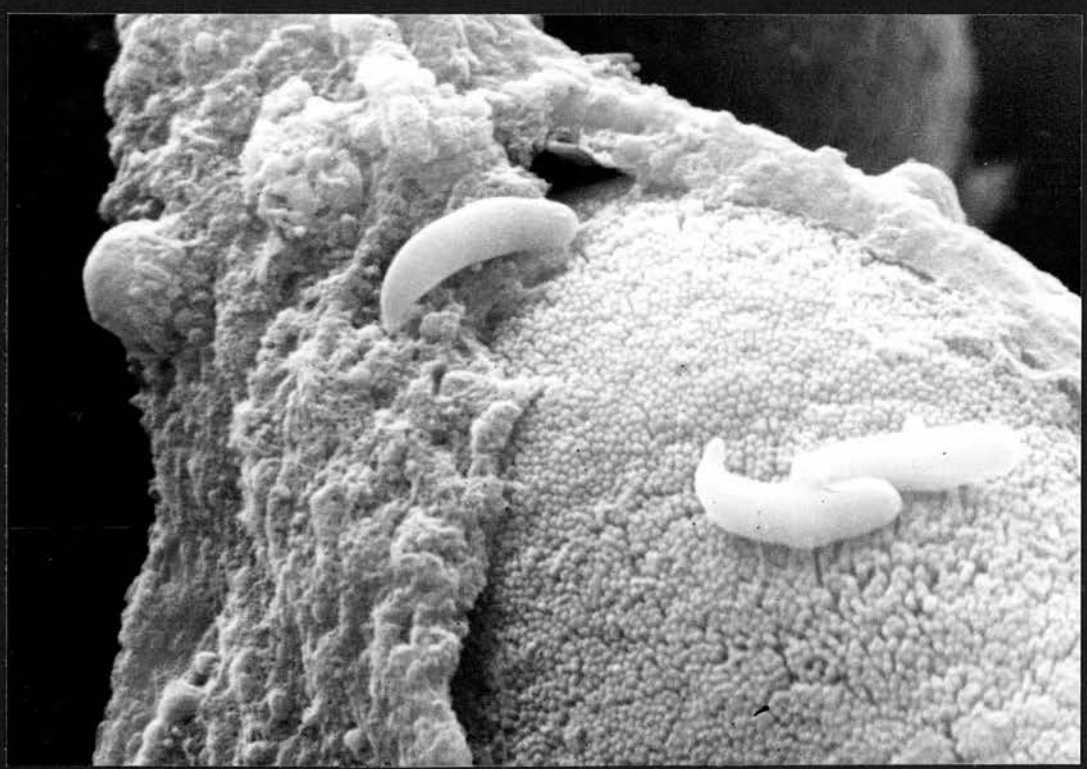


Figure 3.8b x7500

Table 3.5. *In vitro* excystation

	Primary incubation 37°C, 60 minutes			Secondary incubation 37°C, 40 minutes				
						%Ex** with time(min)		
Trial	Reagent*	pH	%Ex**	Reagent	pH	10	20	40
1.	HBSS	6.7	0	HBSS	6.7	0	0	<5
2.	HBSS	6.3	0	HBSS	8.3	0	0	<5
3.	HBSS	4.9	0	HBSS	7.6	0	5	32
4.	0.5% trypsin	6.3	0	HBSS	8.3	0	0	<5
5.	0.5% trypsin	6.3	0	HBSS	6.7	0	0	<5
6.	HBSS	6.3	0	0.1% SDC	8.3	0	0	<5
7.	1.0% trypsin	6.5	0	0.1% SDC	7.0	0	0	<5
8.	1.0% trypsin	6.5	0	0.1% SDC	8.0	0	0	<5
9.	1.0% trypsin	6.5	0	0.1% SDC	9.0	0	0	<5
10.	0.5% trypsin	6.3	0	0.1% SDC	8.0	0	0	<5
11.	1.0% trypsin	6.5	0	1.0% trypsin, 0.1% SDC	7.0	0	0	<5
12.	1.0% trypsin	6.5	0	1.0% trypsin, 0.1% SDC	8.0	0	0	<5
13.	1.0% trypsin	6.5	0	1.0% trypsin, 0.1% SDC	9.0	0	0	<5
14.	HBSS	4.9	0	0.05% SDC	7.6	53	80	80
15.	1.0% trypsin	4.9	0	1.0% trypsin, 0.1% SDC	7.6	67	98	95
16.	1.0% trypsin	4.9	0	0.05% SDC	7.7	48	84	90
17.	1.0% trypsin	3.6	0	0.05% SDC	8.4	83	94	93
18.	Primary incubation omitted			0.05% SDC	8.4	28	37	92

* Oocycts were incubated in Hank's balanced salt solution (HBSS) alone or HBSS containing one or both of trypsin and 7-deoxycholic acid (SDC). Concentrations are on a w/v basis.

** Results were assessed on the basis of excystation percentage (Chapter 2.4).

In vitro excystation

The effects on excystation, of varying the pH conditions and the concentrations of trypsin and 7-deoxycholic acid are shown in Table 3.5. Trials 1-3, where oocysts were incubated only in HBSS, demonstrated some dependency on a pH shift from below 5 in the primary incubation to above 7 in the secondary incubation. Trials 4-13 indicated that, despite varying reagent concentrations and increasing pH above 7 in the secondary incubation, excystation was not triggered if pH in the first stage was greater than 6.

Trial 14 demonstrated the dual triggering mechanism of a pH shift from below 5 to above 7 and incubation with a low concentration of 7-deoxycholic acid. The addition of trypsin under the same pH conditions (trials 15 and 16) did not appear to alter the result.

Increasing the size of the pH change between primary and secondary incubations (trial 17) increased the rate of excystation as shown at 10 minutes. The positive effect of the first stage incubation in trial 17 is indirectly shown in trial 18 where early excystation appeared to be negated in its absence. The sporozoite/shell ratios for trial 17 at the end of first stage incubation and at 10, 20 and 40 minutes after commencement of second stage incubation were 0, 3.4, 3.9 and 3.6 respectively.

Discussion

The following conditions, which were found to accelerate *in vitro* excystation were probably important to *in vivo* excystation:

1. A primary incubation (37^{0}C) at a pH of approximately 4.9 for

60 minutes which in itself did not trigger excystation.

2. A pH shift from 4.9 to at least 7.6 between primary and secondary incubations which triggered rapid excystation especially in the presence of 7-deoxycholic acid.

The former was probably provided by passage through the host's stomach and the latter on entry to the small intestine at a point where the combined conditions of pH and bile salt concentration were optimal. This pH shift appeared to be important to excystation and its effect was enhanced by the presence of 7-deoxycholic acid alone. Whether this effect could be produced with other bile salts was not investigated.

The predominance of endogenous stages in the ileum of the mouse (Experiment 3.1) and the inverse relationship between the distribution of recovered oocysts and sporozoites in the intestine suggested that excystation conditions were more favourable in the distal small intestine. If excystation occurred to the same extent throughout the small intestine then recovery of larger numbers of sporozoites would have been expected from proximal segments.

An estimated 40% of dosed oocysts were accounted for in intestinal flushings over the 5 hour post infection period. Losses may have occurred in the following ways:

1. Oocysts excysting between sampling times and sporozoites penetrating host cells.

2. While oocysts and sporozoites were respectively bright and of characteristic shape on phase contrast microscopy, debris included with intestinal washings may have resulted in some being missed.

3. The inoculating volume (100µl) may have resulted in some of the oocysts being flushed directly into the intestine but the low counts at 1/4 hour after infection indicated that this was not an important problem.

4. The close association of numerous sporozoites with surface mucus in the distal small intestine suggested that mucus trapping may have prevented successful flushing in these segments.

In contrast to mice, investigation of *in vivo* excystation in rat pups was hindered by dislodged sheets of mucus which bound numerous oocysts and sporozoites in all segments of the gut. It was difficult to disperse the mucus without damaging sporozoites and reliable haemocytometer counts could not be made. This finding raised the possibility that, in the rat, oocysts entering the proximal small intestine were trapped there by mucus and eventually excysted. Infection of the proximal small intestine in this way could be one explanation of the distribution of histopathological observations in the rat.

The excystation method described in trial 17 (Table 3.5) was subsequently used in all *in vivo* and *in vitro* experiments requiring sporozoites. It gave rapid, high percentage excystation with minimal exposure of sporozoites to potentially damaging agents like trypsin and bile salts. Since *Cryptosporidium* oocysts contain 4 sporozoites, the theoretical value for the sporozoite/shell ratio would also be 4. In practice, values between 3 and 4 were normally found using the excystation conditions and methods of assessment described in this experiment.

EXPERIMENT 3.4. STUDIES OF MEROZOITES IN SURFACE MUCUS

Introduction

Experiments 3.1 and 3.3 showed that, in the mouse small intestine, the bulk of excystation and subsequent development of endogenous stages occurred in the ileum. This presented the opportunity to study the development of endogenous stages in a restricted section of small intestine.

The aims of this experiment were:

1. to improve understanding of the parasite's life cycle, especially in terms of asexual reproductive capacity and

2. to examine the association between merozoites and surface mucus.

Materials and methods

Swiss White mice, used throughout this experiment, were orally infected with 10^6 oocysts (cervine isolate) at 5 days of age. As with other mouse strains used in Experiment 3.1, histopathological examination of the infection over an 11 day post infection period showed that the bulk of endogenous stages were located between the 80 and 100% small intestinal intervals. Accordingly, the last 20% of small intestine (approximately 20-30mm) was used for studies of endogenous stages and surface mucus throughout this experiment. The methods described in Chapters 2.7.2 and 2.10 were used to collect merozoites and estimate the mucus content of intestinal washings.

Results

Three days after infection numerous merozoites were seen in both wet mounts of ileal surface mucus examined by phase contrast microscopy and in surface mucus of the ileum and colon examined in Giemsa stained paraffin sections (Figure 3.9).

Collection of oocysts and merozoites

Figure 3.10 shows the number of merozoites and oocysts collected per mouse in ileal washings over an 11 day post infection period. There was a rapid increase in the number of merozoites collected over the first 3 days of infection after which numbers remained high for the next 5 days. From 9-11 days after infection the number of merozoites collected steadily declined. Oocysts were not detected until day 4 after infection. In contrast to control mice, numerous strands of mucus were dislodged from infected ilea which imparted a slight cloudiness to the PBS. This was evident from 3-4 days after infection.

Mucus estimation

The amount of mucus present in ileal wash fluid was estimated by measurement of hexose content, both in the whole preparation and in the supernate. Changes in the estimated mucus collected from the ilea of age-matched control and infected mice, are shown over the first 8 days of infection in Figure 3.11. Control values remained relatively steady throughout the experiment. Values in the infected group were similar to controls until day 4 when they increased and remained elevated throughout the remainder of the experiment.

Discussion

In this experiment, the oocyst prepatent period for *Cryptosporidium* in mice was 4 days. This closely agreed with

Figure 3.9. Mouse colonic mucosa on day 3 after infection with 10^6 *Cryptosporidium* oocysts. Endogenous stages were attached to enterocytes (large arrow) and numerous merozoites were present in surface mucus (small arrow). Giemsa stain. x1600.

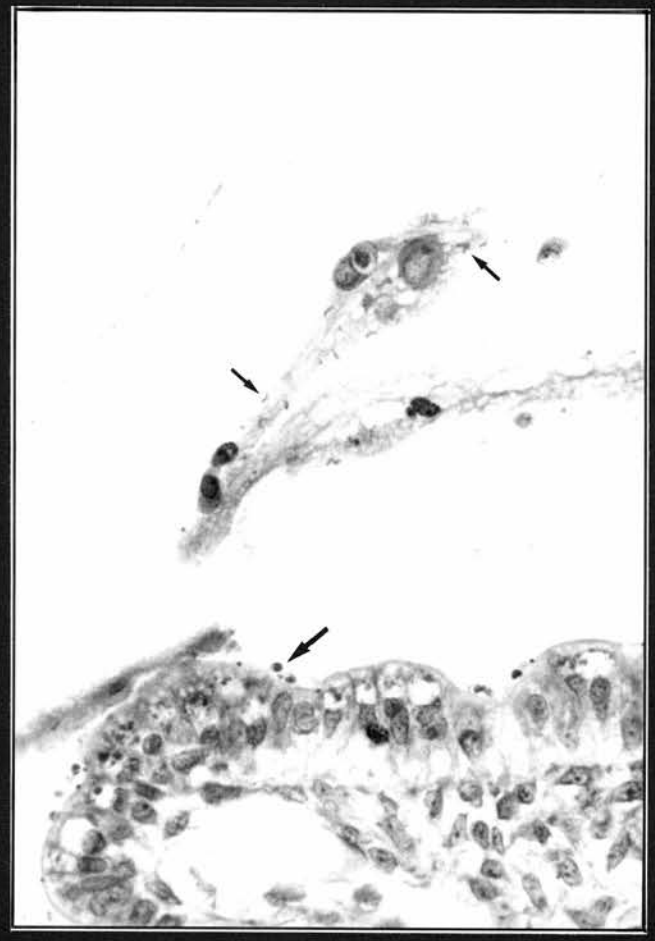


Figure 3.10. Merozoites (——) and oocysts (- - -) collected in mouse ileal washings, with phosphate buffered saline, over an 11 day post infection period. Mice were infected with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 5 days of age. Oocysts were not counted beyond 8 days after infection. Values represent the mean of measurements from 8 mice.

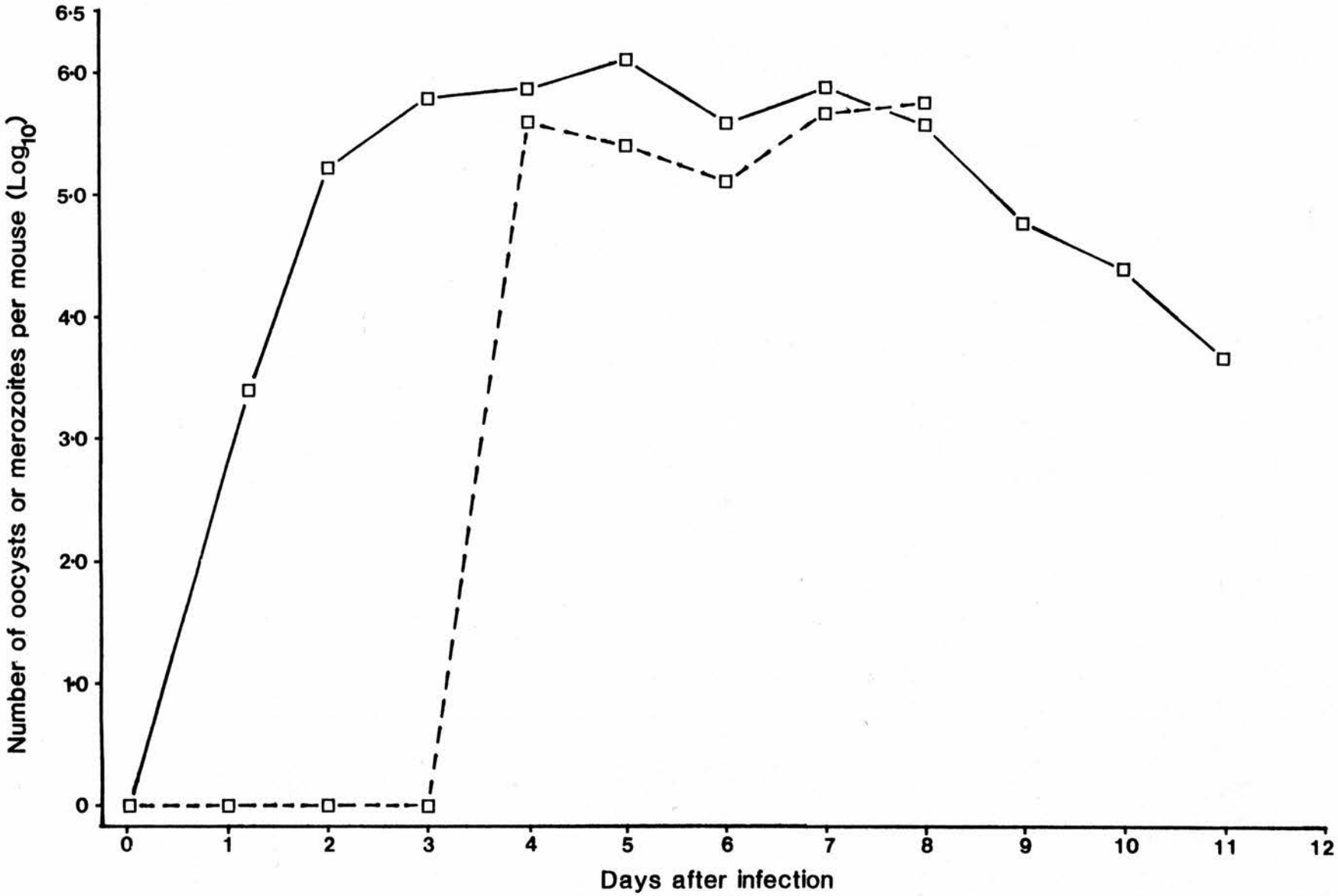

Number of oocysts or merozoites per mouse (Log10)
6·5
6·0
5·0
4·0
3·0
2·0
1·0
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Days after infection

Figure 3.11. Estimation of the mucus content, of ileal wash fluid from mice infected with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 5 days of age. The mucus content was estimated by hexose analysis using D-galactose as the standard. Analysis of whole wash fluids (○) and their supernates (●) are shown, from both principal (- - -) and age-matched control (——) mice. Significant differences (Student's t-test) between principal and control mice are shown at the bottom of the figure (ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Values represent the mean (±SEM) of measurements from 10 mice.

Current and Reese (1986), who observed oocysts in histological sections of mouse ileum from 3 days post infection, and Heine *et al* (1984a) who found a prepatent period of 4 days in mice.

In the present experiment, the merozoites observed in ileal washings from day one after infection appeared more slender and less comma shaped than *in vitro* excysted sporozoites. Over days 1-3 post infection the number of merozoites collected per mouse increased over 100 fold. The magnitude of this increase may be accounted for by proliferation of asexual generations. It approximates to the theoretical asexual expansion factor of 128; that is one oocyst yielding four sporozoites, each of which produce 32 merozoites through type(1) and type(2) schizont generations.

The number of merozoites collected from each mouse ileum remained at the three day post infection level for the next five days. While this experiment did not take account of the time merozoites might remain in surface mucus, the results suggest sustained asexual reproduction from day 3 to 8 after infection. Cyclic development of type(1) merozoites (Current and Long, 1983) and possibly type(2) merozoites may have accounted for this sustained production. Thin-walled oocysts, observed in infected mice by Current and Reese (1986), may have contributed to continuing merozoite production but were not investigated in this experiment.

Macroscopic evidence of increasing amounts of dislodged mucus from day 3-4 after infection onwards was supported by estimates of mucus in the ileal wash media. Explanations for these observations might include the following:

1. Infected ilea did develop a thicker layer of mucus. Although increased numbers of goblet cells were not a feature on histopathological examination of infected ilea, their rate of secretion may have increased.

2. Mucus may have been more easily dislodged if its viscosity and gel forming properties had been altered as a result of the infection.

3. Estimation of mucus may have been influenced by the carbohydrate content of any inflammatory effusion on the surface of infected ilea. The constituents of an inflammatory effusion reflect those of the plasma and many plasma proteins are glycoproteins. The dry weight of mucus glycoprotein is at least 80% carbohydrate whereas that of a typical plasma glycoprotein (plasma globulin) is less than 25% (Allen *et al*, 1982; Clamp, 1986). Hence, changes in the carbohydrate content of ileal washings were more likely to have represented changes in the quantity of dislodged mucus in infected mice.

EXPERIMENT 3.5. STUDIES IN NEONATAL LAMBS

Introduction

It has been shown in gnotobiotic lambs that *Cryptosporidium* does cause diarrhoea in the absence of normal intestinal microflora (Snodgrass *et al*, 1984). Whether the presence of a normal intestinal microflora influences the magnitude or duration of oocyst shedding has not been investigated. These and other aspects of cryptosporidiosis were examined in colostrum deprived conventional and gnotobiotic lambs.

Materials and methods

Seventeen colostrum deprived conventional and sixteen gnotobiotic lambs, derived as described in Chapter 2.2.2, were infected orally with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 5 days of age. Preinoculation faecal and blood samples were collected from all lambs at 5 days of age. Thereafter, daily sampling continued until 17 days post infection. Samples of faeces and blood were collected weekly, from 3-6 weeks post infection, from colostrum deprived conventional lambs. The methods of infection, collection and preparation of ante- and post-mortem specimens are described in Chapters 2.5, 2.6 and 2.7 respectively.

Gnotobiotic lambs were infected as two consecutive groups:

I. six principal and four control lambs killed 16 days after infection (21 days of age)

II. four principal and two control lambs killed 8 days after infection (13 days of age).

Experimental methods were the same for both groups with lambs housed in pairs in isolators.

Results

Bacteriology

Colostrum deprived lambs

Inocula were not cultured for bacteria. Of the 7 lambs from which blood was cultured at one day of age, 5 showed no growth. An unidentified Gram negative rod was cultured from one lamb while *Escherichia coli* and *Streptococcus* sp. were cultured from the other. Both lambs remained clinically normal despite bacteraemia.

Gnotobiotic lambs

No bacteria were cultured from ethanol-treated inocula or from lamb faecal swabs taken at birth. The results of culturing faecal swabs after birth were as follows:

Group I. The ten lambs (4 controls, 6 principals) which were killed at 21 days of age all eventually became contaminated with either *Streptococcus* sp. or *Bacillus* sp.. *Streptococcus* sp. was cultured from the two control pairs at 3 and 16 days of age respectively. All principal lambs were culture negative at 5 days of age when inoculated with *Cryptosporidium*. Either *Streptococcus* sp. or *Bacillus* sp. was cultured from two principal pairs at 8 days and from the remaining principal pair at 16 days of age. Bacteria were not speciated and no medication was given to eliminate this contamination.

Past experience with the type of milk carton passed into the gnotobiotic isolators has shown that *Streptococcus* sp. and *Bacillus* sp. can be surface contaminants (Snodgrass *et al*, 1984; Bell, personal communication). The accidental contamination of this group probably resulted from inefficient

surface sterilization of the milk cartons before entry into the isolator. For ease of distinction these lambs will continue to be denoted as Group I gnotobiotic.

Group II. The six lambs (2 controls, 4 principals) which were killed at 13 days of age remained culture negative, probably because entry into the isolators was minimized and greater attention paid to surface sterilization of milk cartons.

Clinical signs

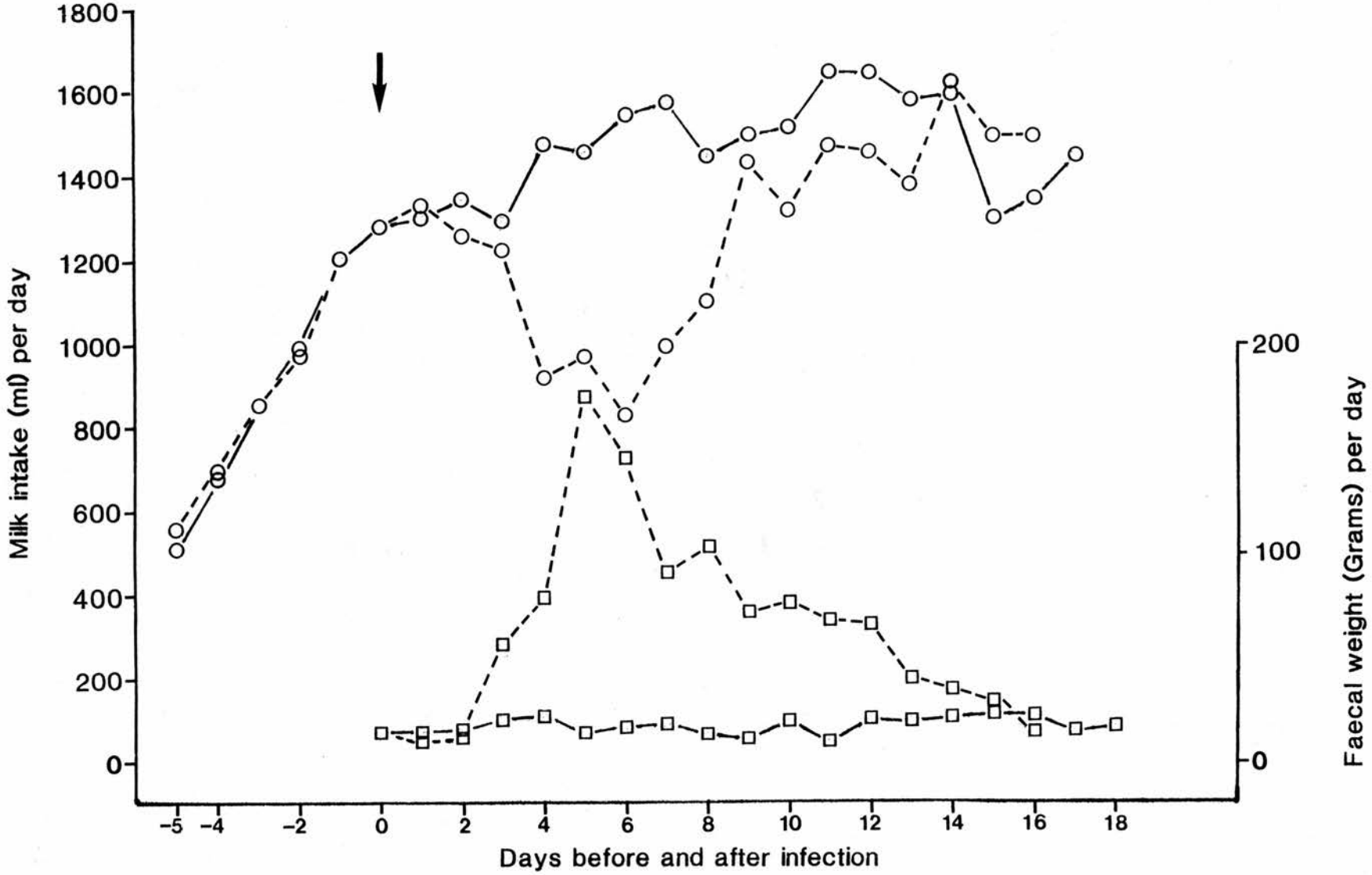
Clinical signs were similar for both colostrum deprived and gnotobiotic lambs. Infected lambs were clinically normal and their faeces remained of normal pasty consistency for the first 3-4 days post infection. After this, there was diarrhoea for a variable period up to 15 days post infection, whereafter faeces became consistently firm. The severity of diarrhoea varied between lambs from mild to profuse and watery. Between 4 and 10 days after infection lambs were anorexic and lethargic. They were often eager to feed but quickly lost interest after sucking a small quantity of milk. Group mean daily milk intake, used to assess appetite, and group mean daily faecal weight are shown in Figure 3.12 for control and principal gnotobiotic lambs. During 3-13 days after infection, depressed group mean daily milk intakes were associated with elevated group mean faecal weights when compared with control animals.

Oocyst shedding

Colostrum deprived lambs

Of the 15 principal lambs, oocysts were first detected in faeces at 3 (2 lambs), 4 (10 lambs) or 5 (3 lambs) days after infection. The oocysts from both lambs which began excreting 3 days after infection were detected on phenol auramine stained

Figure 3.12. Group mean daily milk intake, (○,males and females) and group mean daily faecal weight (□,only males) of gnotobiotic lambs infected (- - -) with 10^6 *Cryptosporidium* oocysts at 5 days of age(↓) and age-matched controls (——). Mean values were calculated from measurements on control (2 females, 2 males) and principal (6 males) Group I gnotobiotic lambs.


1800
1600
1400
1200
1000
800
600
400
200
0
Milk intake (ml) per day
200
100
0
Faecal weight (Grams) per day
-5
-4
-2
0
2
4
6
8
10
12
14
16
18
Days before and after infection

faecal smears but oocyst numbers were too low to be detected in dilutions of faeces examined in a haemocytometer. Faecal oocyst concentrations fluctuated between 10^5 and 10^7 per gram and fell below detectable levels by day 16 after infection. Total daily oocyst output, as measured in male lambs, varied from 10^7 to $10^{9.1}$ over the 4-10 day post infection period (Figure 3.13). Over this period the daily oocyst output from each of the 7 principal male lambs exceeded 10^9 on two occasions, both in the same animal. The mean cumulative number of oocysts produced by each of the male lambs over the first 16 days of infection was 2.9×10^9. Oocysts were never detected in the faeces of the 2 control male lambs.

Gnotobiotic lambs

Oocysts were first detected 3 (6 lambs) or 4 (4 lambs) days after infection. For the 10 male lambs in Groups I and II, the fluctuations observed in faecal oocyst concentration were similar to those seen in colostrum deprived lambs. At necropsy, 16 days after infection, oocysts were detected in the faeces of two of the six principal lambs from Group I. Total daily oocyst output varied from 10^7 to $10^{9.9}$ over the 4-10 day post infection period. Over this period the daily oocyst output from each of the six principal male lambs in Group I exceeded 10^9 on 15 occasions, involving all animals (Figure 3.13). The mean cumulative number of oocysts produced by each of the six male lambs from Group I over the first 16 days of infection was 8.1×10^9.

Mucosal pathology

Intestine from principal and control gnotobiotic lambs was examined by light and scanning electron microscopy.

Figure 3.13a,b. Group mean oocyst excretion expressed as $\log_{10}$ total oocysts/day/lamb (±SEM) from conventional and gnotobiotic male lambs infected with 10^6 *Cryptosporidium* oocysts at 5 days of age. Oocyst excretion profiles from six Group I(△) and four Group II(▲) gnotobiotic lambs (3.13a) and seven conventional lambs (○, 3.13b) are shown. Oocysts were never detected in the faeces of conventional or gnotobiotic control lambs.

Figure 3.13a

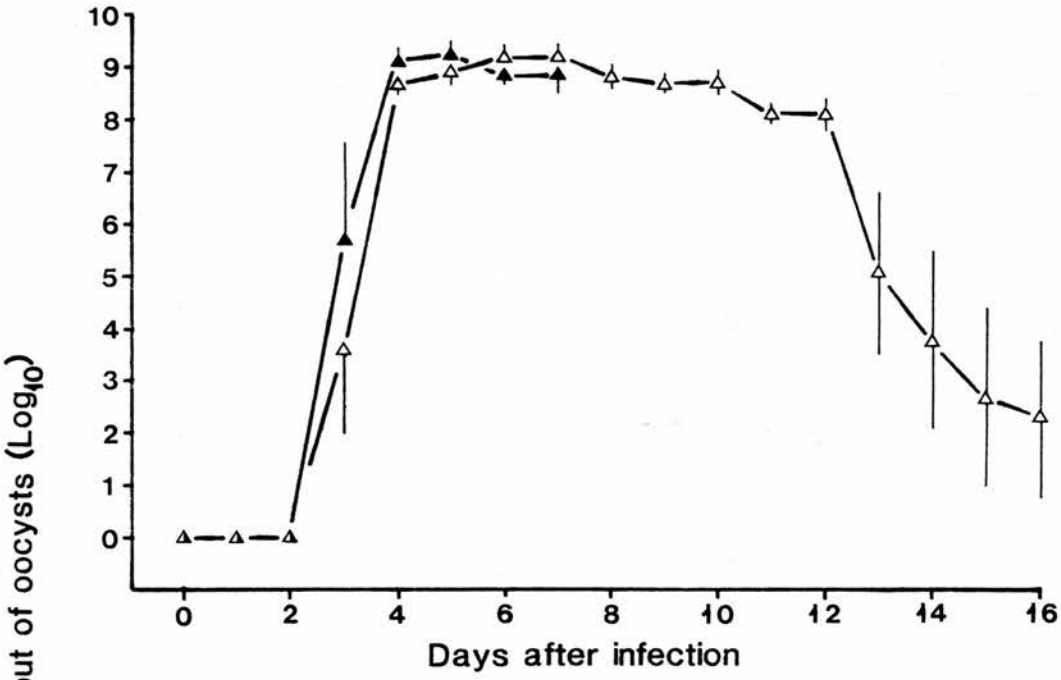
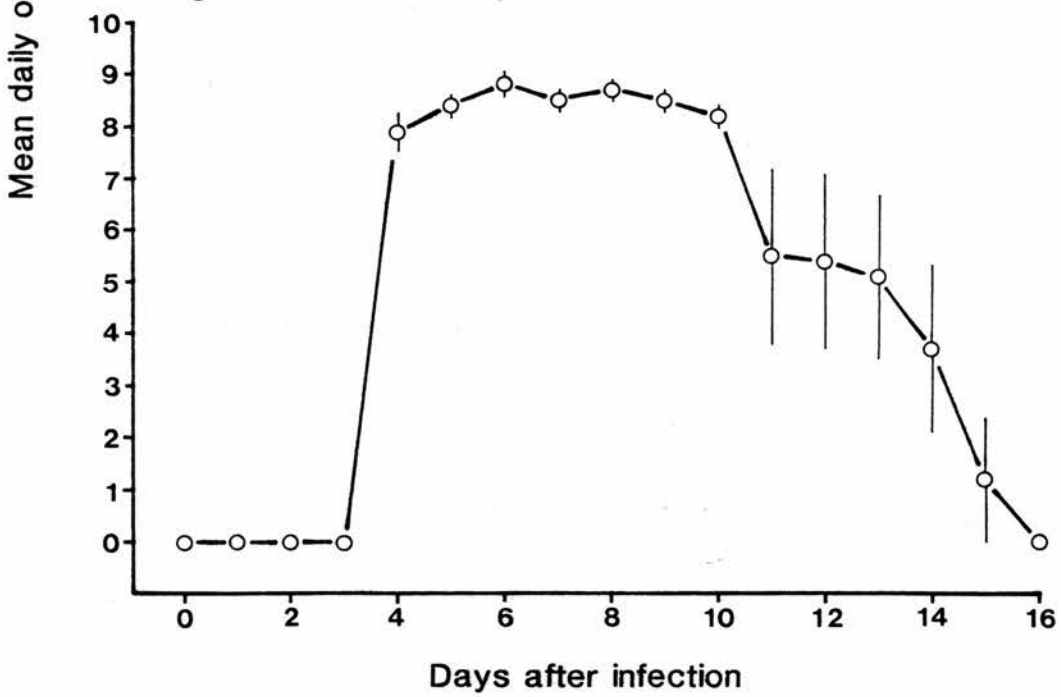


Figure 3.13b



Cryptosporidium endogenous stage population density

Eight days after infection endogenous stages were detected throughout the small and large intestine, with the majority located in the distal small intestine, caecum and colon. Sixteen days after infection endogenous stages were detected in small numbers in the caecum and colon only.

Large numbers of endogenous stages were attached to the epithelium of the bile duct as it passed through the wall of the duodenum. This was an incidental finding in one lamb at 16 days after infection and 2 lambs at 8 days after infection.

Histological changes in the intestine

Eight days after infection villi in the distal small intestine were stunted and the crypts appeared elongated. Normal villous epithelium had been replaced by a low columnar or cuboidal type to which numerous endogenous stages were attached. Endogenous stages were also observed on epithelial bridges which fused adjacent villi (Figure 3.14). The lamina propria was infiltrated by large numbers of lymphocytes and a few macrophages. Numerous granulocytes (neutrophils and eosinophils) were also present in the lamina propria but in numbers similar to those seen in control animals. Eight days after infection the caecal and colonic mucous membranes were thickened; glands were elongated and often contained cell debris, especially degenerating granulocytes. Cellular infiltration of the lamina propria was similar to that in the small intestine. By 16 days after infection normal morphology had been restored in both small and large intestine.

Examination of the small intestine 8 days after infection, using scanning electron microscopy, showed that endogenous stages were overlaid by a blanket of surface mucus (Figure 3.14). The weights of crude surface mucus collected from each intestinal

Figure 3.14a,b. Scanning electron micrographs of the ileum from a gnotobiotic lamb 8 days after infection with 10^6 *Cryptosporidium* oocysts.

3.14a. The tips of two fused, atrophic villi are shown. Numerous endogenous stages (arrows) are attached to the epithelium on the villi and the bridge connecting them. x1125.

3.14b. A villous tip covered by a blanket of mucus beneath which numerous endogenous stages can be seen (arrows). x1500.

Figure 3.14

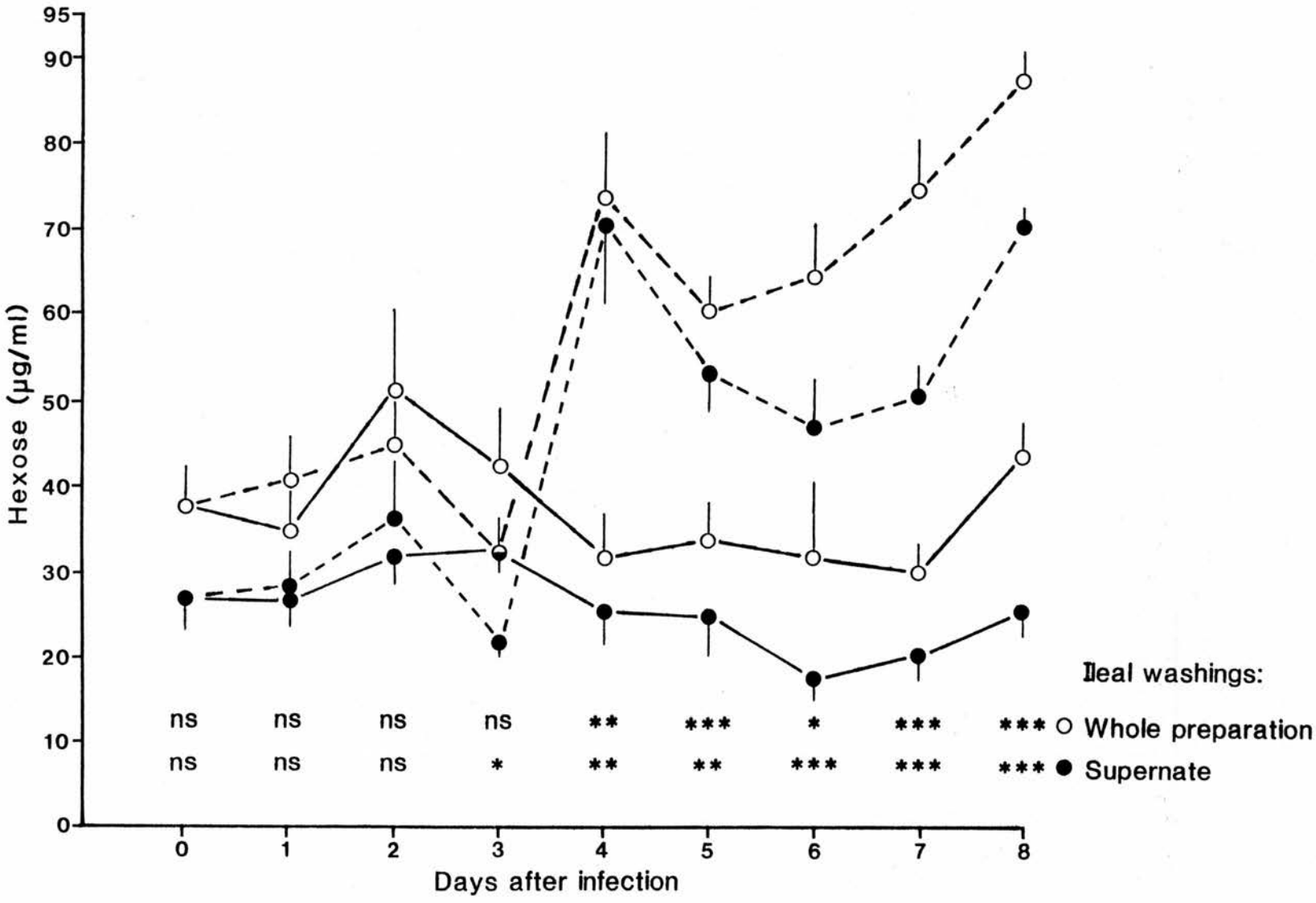

Hexose (µg/ml)
95
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Days after infection
ns ns ns ns ** *** * *** ***
ns ns ns * ** ** *** *** ***
Ileal washings:
Whole preparation
Supernate

Figure 3.14a

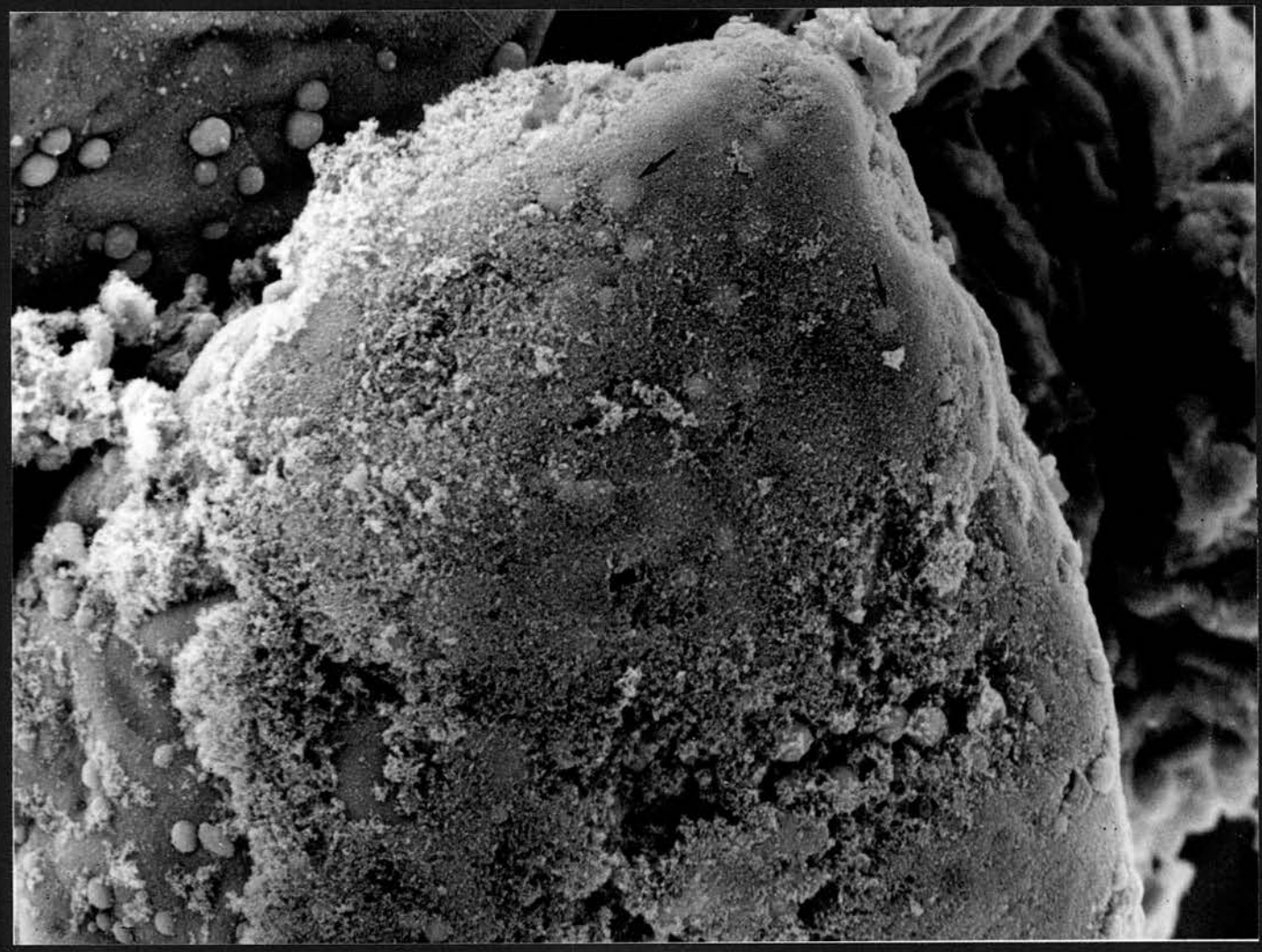
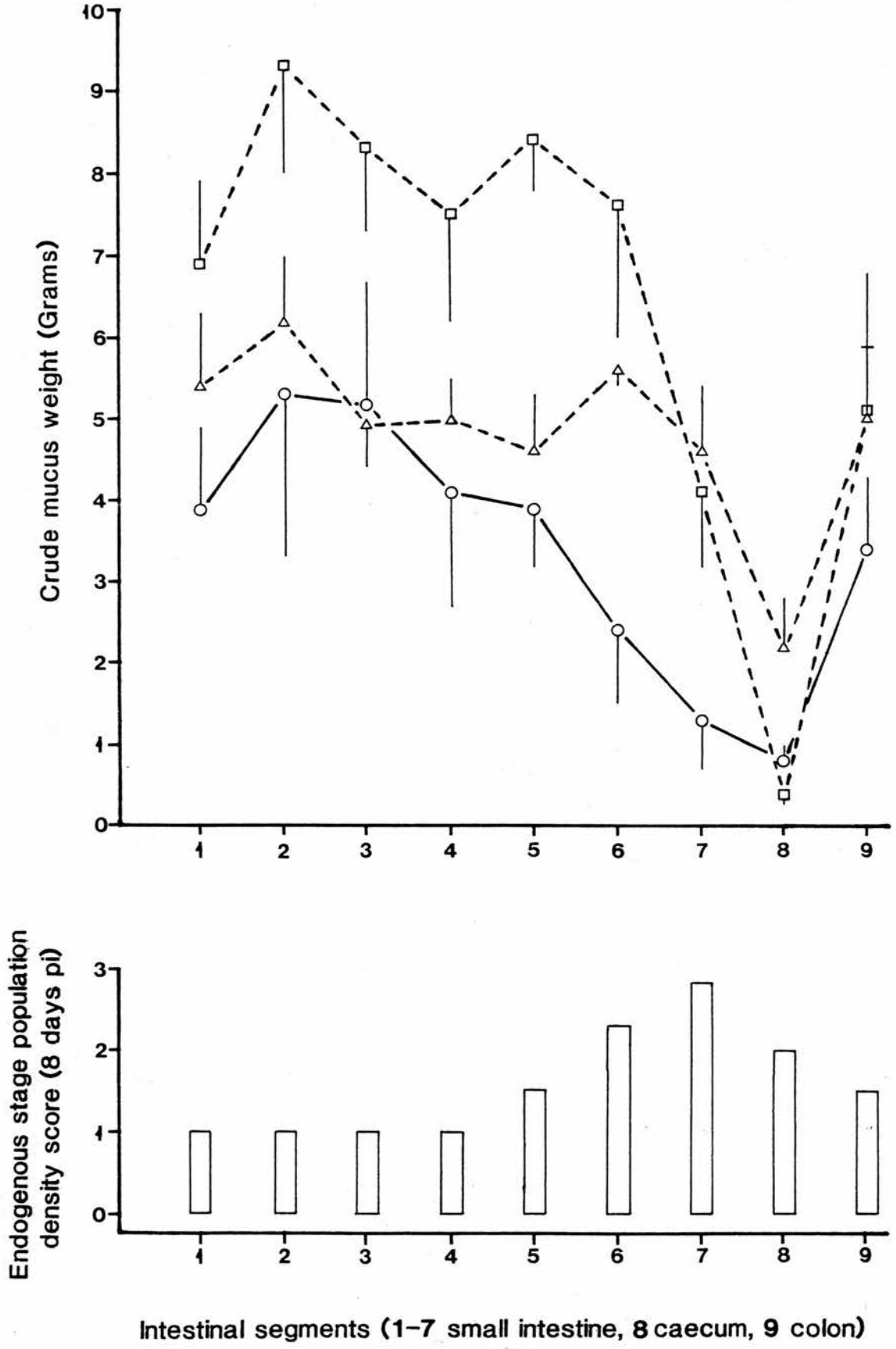


Figure 3.14b

Figure 3.15. Group mean weights of crude mucus collected off intestinal segments from gnotobiotic lambs at 8 (△- - -△) and 16 (□- - -□) days after infection with 10^6 *Cryptosporidium* oocysts at 5 days of age and their age-matched pooled controls (○——○). Endogenous stage population density 8 days after infection is shown at the bottom of the figure for each intestinal segment. Intestinal segments 1-7 represent duodenum to ileum respectively. Mean values were calculated on measurements from 4 (8 days after infection), 6 (16 days after infection) and 6 (pooled control) lambs.


Crude mucus weight (Grams)
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Endogenous stage population density score (8 days pi)
3
2
1
0
1
2
3
4
5
6
7
8
9
Intestinal segments (1–7 small intestine, 8 caecum, 9 colon)

segment are shown in Figure 3.15. Compared to controls, greater quantities of crude mucus were collected from segments 5,6 and 7 ($p<0.05$) at 16 days after infection and from segments 6,7 and 8 ($p<0.05$) at 8 days after infection. Within two hours of collection into PBS at 4^0C this mucus had formed a viscous gel which moved as a single body when the containing vessel was tilted. The gel reformed after vigorous vortex mixing and was most evident in specimens collected from intestinal segments 5-9, where larger numbers of endogenous stages were located.

Discussion

The inocula for colostrum deprived conventional and gnotobiotic lambs were not examined for rotavirus. However, inocula were disinfected with ethanol (Chapter 2.5.1) and this has been shown to be effective against rotavirus (Sattar, Raphael and Springthorpe, 1983). Group I gnotobiotic lambs became contaminated with *Streptococcus* sp. or *Bacillus* sp. during the experiment but coliforms and anaerobic bacteria, commonly associated with the intestine, were not isolated on culture. The oocyst excretion profiles of Group I and II gnotobiotic lambs were similar over the first week of infection.

The prepatent period and clinical signs observed here for conventional and gnotobiotic lambs were the same as those reported previously for gnotobiotic lambs (Snodgrass *et al*, 1984). Although total daily oocyst output was similar between lambs, oocyst concentrations varied greatly, from 10^5-10^7 per gram, since faecal volumes varied with the severity of the diarrhoea.

On average each gnotobiotic lamb produced about 2.8 times as many oocysts over the experiment as each conventional lamb and frequently shed more than 10^9 oocysts/day. This suggested that limitations on the parasite's reproductive potential were less in gnotobiotic compared to conventional lambs. Although Group I

gnotobiotic lambs became contaminated, a more extensive bacterial flora would have been expected in the conventional lambs. Whether it was the presence of a normal gastrointestinal microflora which limited the reproductive potential of the parasite in conventional lambs has not been investigated.

The finding of large numbers of endogenous stages attached to bile duct epithelium in a lamb 16 days after infection occurred at a time when small numbers were detected in caecum and colon but not elsewhere in the intestine. This, and similar incidental findings in lambs 8 days after infection and in an adult rat (Experiment 3.2) suggests that the bile duct may be a site of lingering infection in older animals. Cryptosporidiosis in AIDS patients can involve the biliary duct where it may contribute to chronic crytosporidial enteritis by acting as a reservoir of infection (Soave and Johnson, 1988). Other histopathological changes observed in this experiment were similar to those reported previously in conventional and gnotobiotic lambs (Angus *et al*, 1982b; Snodgrass *et al*, 1984).

A method was developed (Chapter 2.7.1) which gently skimmed crude mucus from the surface of the intestine in a manner which allowed the same action to be applied to all segments from all lambs. Mucus was collected primarily for the purpose of detecting antibody specific for *Cryptosporidium* (Chapters 4 and 5). Because the same collection procedure was used on all intestinal segments a comparison was made of mucus weights between principal and control lambs. Although crude mucus weights included some dislodged villi and surface debris, there were significant differences between infected and control lambs. This result agreed with that of Experiment 3.4 which indicated that the quantity of dislodged mucus increased in infected mice.

CHAPTER 4. Analysis of the kinetics of specific antibody isotypes produced by lambs and rats infected with *Cryptosporidium*

Introduction

The course of cryptosporidiosis in lambs has been described in terms of clinical signs, mucosal pathology and oocyst shedding (Chapter 3, Experiment 3.5). This chapter examines the kinetics of specific antibody isotypes, in serum and extracts of faeces and intestinal mucus, in relation to oocyst shedding. Similarly, the kinetics of serum antibody in the Lister rat model (Chapter 3, Experiment 3.2) are examined in conjunction with endogenous stage population density.

Experimental Design

Lambs

Serum and extracts of faeces and intestinal mucus were obtained from the same colostrum deprived conventional or gnotobiotic lambs described in Experiment 3.5 (Chapter 3). Principal lambs were infected orally with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 5 days of age. The methods of infection, collection and preparation of specimens have been described in Chapter 2, Sections 2.5, 2.6 and 2.7 respectively. Analysis of intestinal mucus has been described in Chapter 2, Section 2.10.

Rats

Serum specimens were obtained from Lister rats, allocated to either control or to one of three principal groups, described in Experiment 3.2 (Chapter 3). Rats were infected either once (at 4 days of age, group 1), three times (at 4, 15 and 26 days of age, group 2) or twice (at 15 and 26 days of age, group 3) with 10^6 *Cryptosporidium* oocysts (cervine isolate) given orally on each

occasion. The methods of infection and collection of serum specimens have been described in Chapter 2, Sections 2.5 and 2.7.

Results

Section 1. Antibody response in lambs

Kinetics

Specific antibody was first detected in sera and faecal extracts of conventional lambs at 7 and 10 days post infection respectively. The group mean serum titre continued to rise until 26 days after infection and then remained steady for the duration of the experiment. Specific coproantibody reached a peak group mean titre in males on day 16 after infection and then declined to undetectable levels by day 36 (Figure 4.1).

Isotype

Specific group mean isotype titres detected in the sera of conventional lambs on days 0, 7, 11 and 18 after infection are shown in Figure 4.2a. An early rise in IgM was accompanied by a slower but steady rise of IgG. A peak titre of IgA was recorded at 11 days after infection. Sera from gnotobiotic lambs were examined on a different time scale; although a similar isotype response was found, IgM antibody was the first to be detected (Figure 4.2b).

Figures 4.3 and 4.4 show the group mean titres of specific immunoglobulins detected in faecal extracts of conventional and gnotobiotic male lambs respectively, in relation to their group mean oocyst shedding. Specific IgA was the only isotype detected in faecal extracts from conventional lambs. It was not detected until day 10 and titres continued to rise until day 16 after infection. There was a similar rise in specific faecal

Figure 4.1. Group mean titres, expressed as $\log_n$ IFA titre (± SEM) of sera (●) and faecal extracts (■) from colostrum deprived conventional lambs infected with 10^6 *Cryptosporidium* oocysts at 5 days of age and uninfected control lambs. Assays were done with FITC-labelled pig antisheep IgG (H+L) conjugate on sera (males and females) and faecal extracts (only males) from control (——) and principal (- - -) lambs. Values represent the mean of 15 principal lambs (7 males, 8 females) and 2 control lambs (both male).

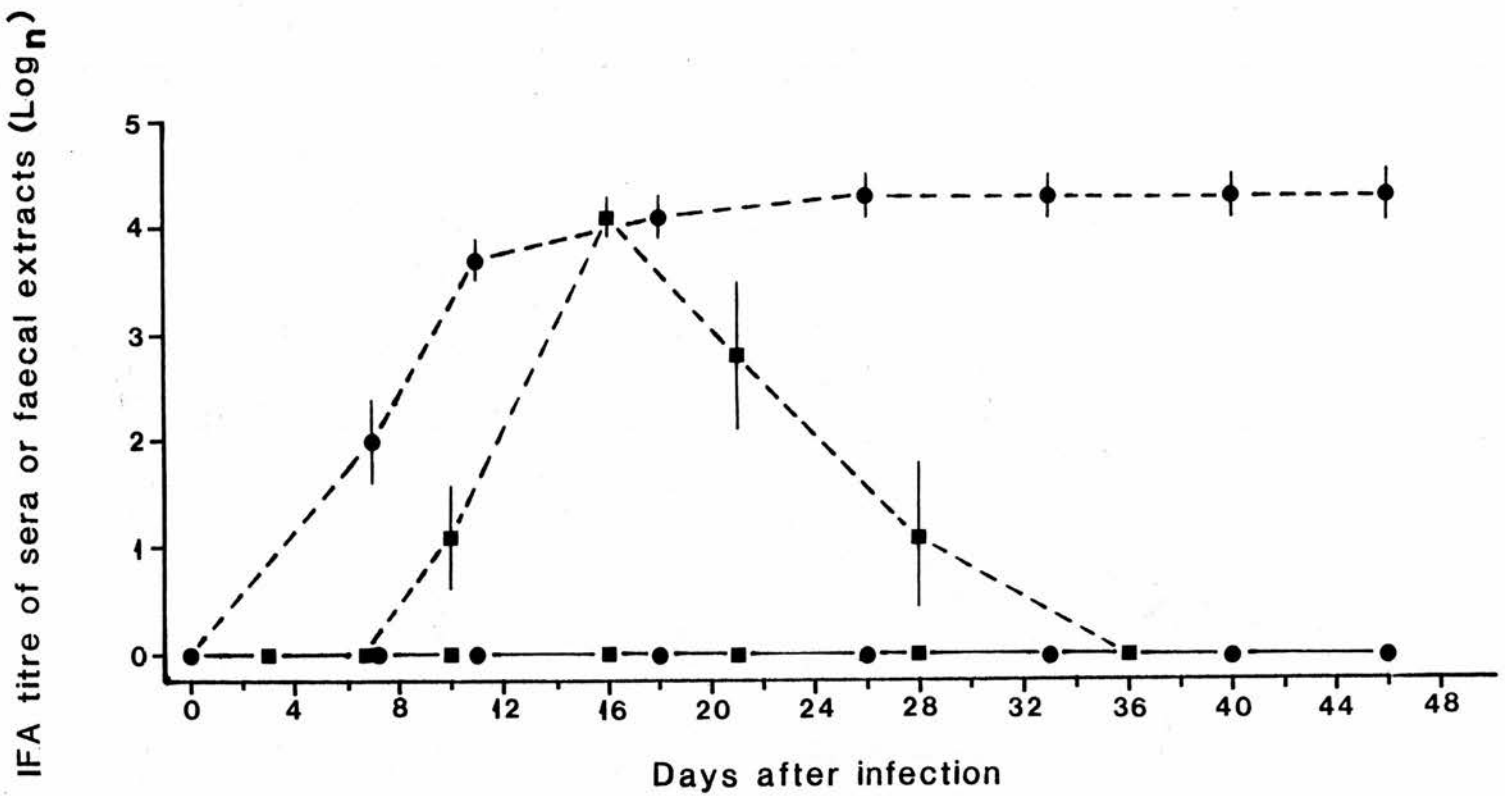

IFA titre of sera or faecal extracts (Log n)
5
4
3
2
1
0
0
4
8
12
16
20
24
28
32
36
40
44
48
Days after infection

Figure 4.2. Group mean serum titres, expressed as $\log_n$ IFA titre (±SEM), from conventional (4.2a) and gnotobiotic (4.2b) lambs infected with 10^6 *Cryptosporidium* oocysts at 5 days of age and uninfected control lambs. Assays were done with FITC-labelled pig anti-sheep IgM (●), IgA (▲) and IgG (■) on sera from control (——) and infected (- - -) lambs. Values represent the mean of 17 conventional (15 principal and 2 control) and 10 gnotobiotic (6 principal and 4 control) lambs.

Figure 4.2a

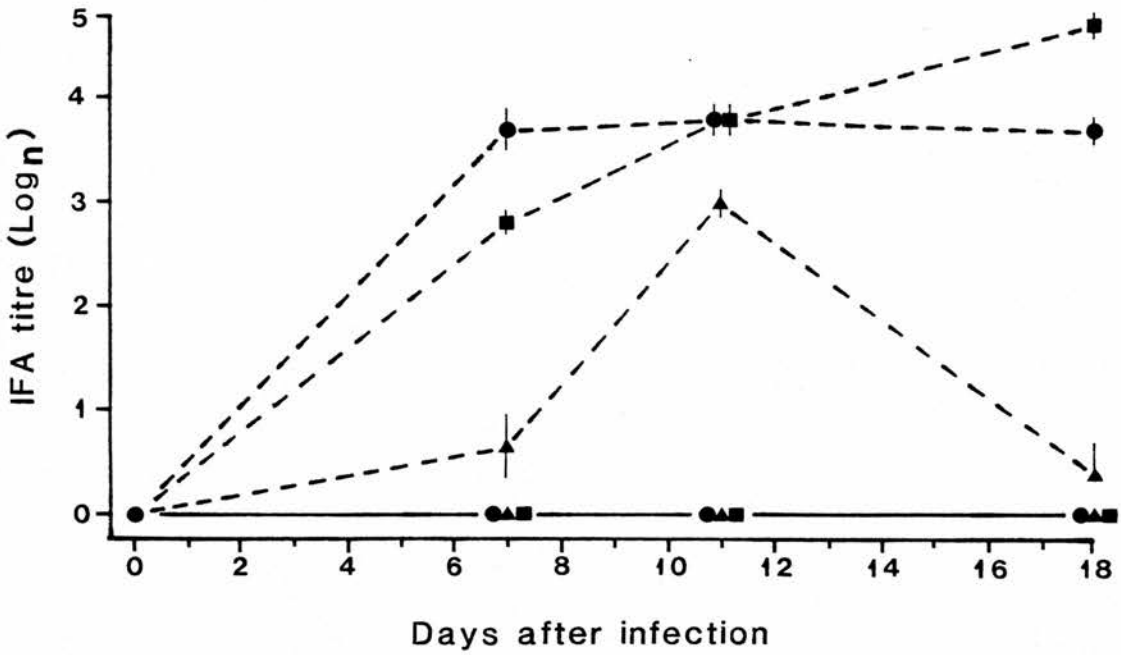


Figure 4.2b

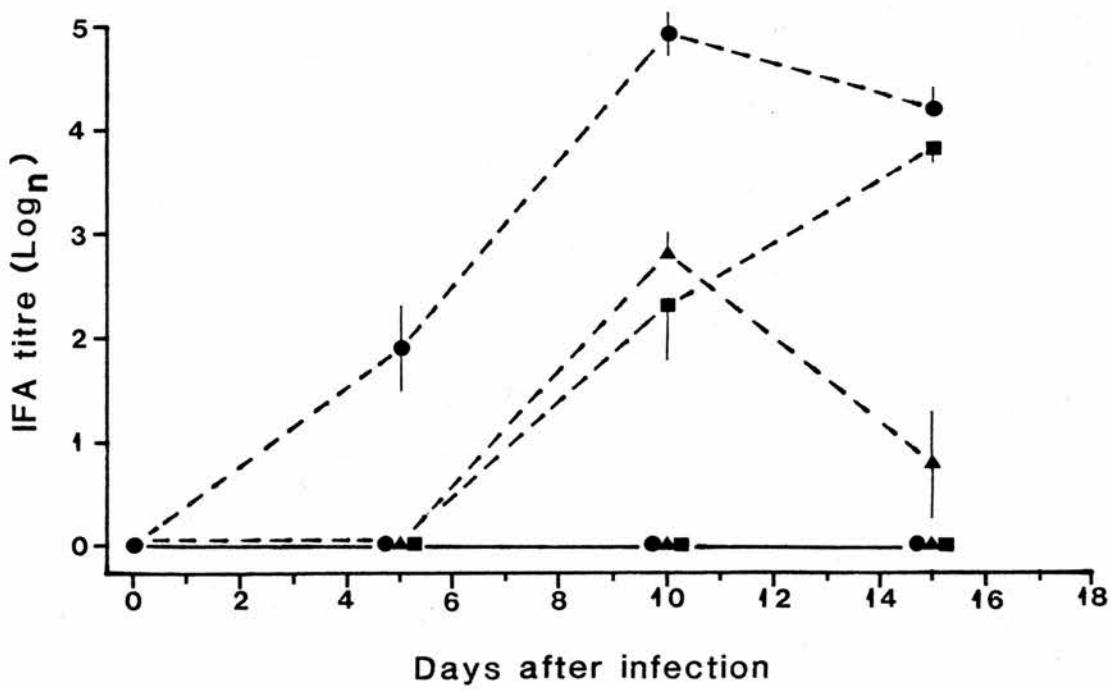


Figure 4.3. Group mean oocyst excretion and faecal IgA titres expressed as $\log_{10}$ total oocysts/day/lamb (○) and $\log_n$ IFA titre (±SEM) (▲) respectively from conventional lambs infected (- - -) with 10^6 *Cryptosporidium* oocysts at 5 days of age and uninfected control (——) lambs. Values represent the mean of measurements from 7 principal and 2 control male lambs.

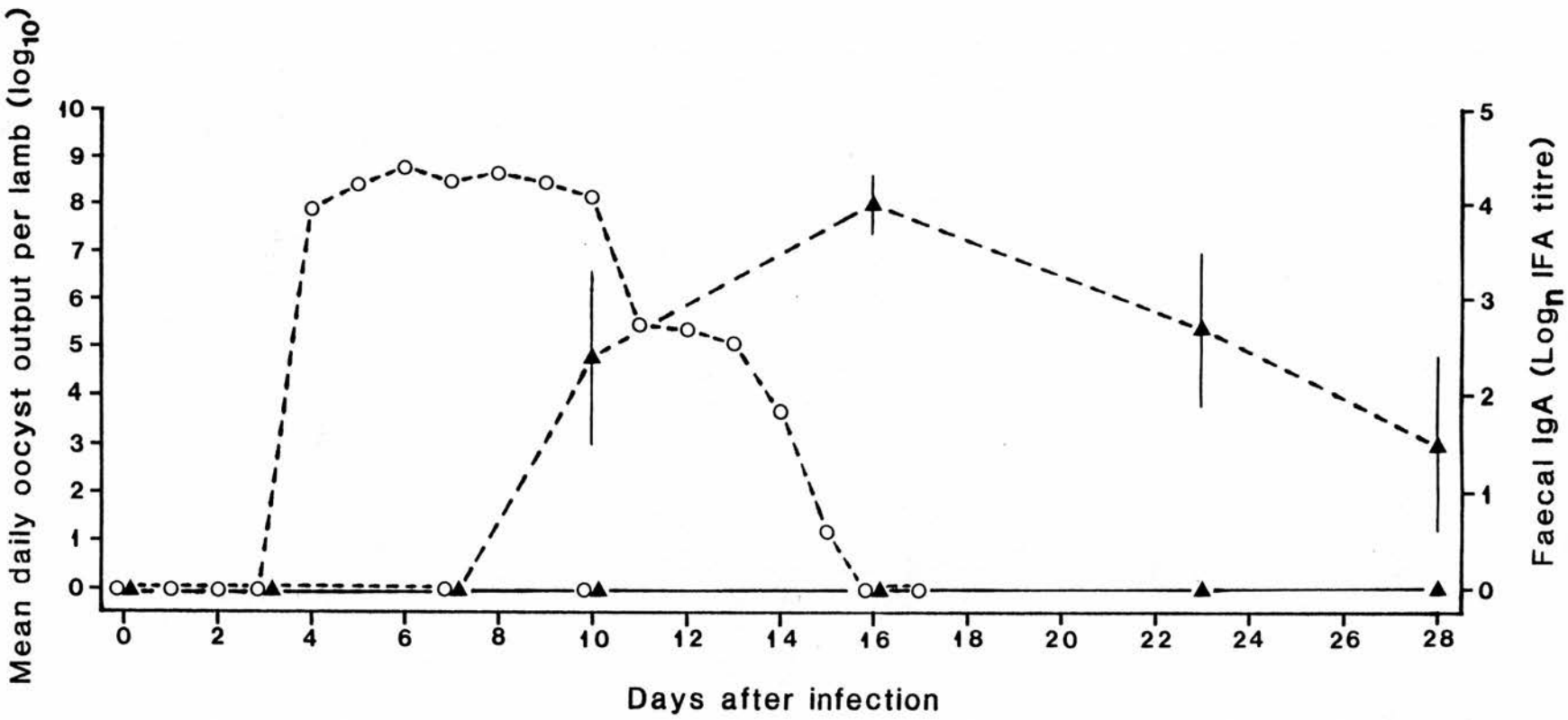
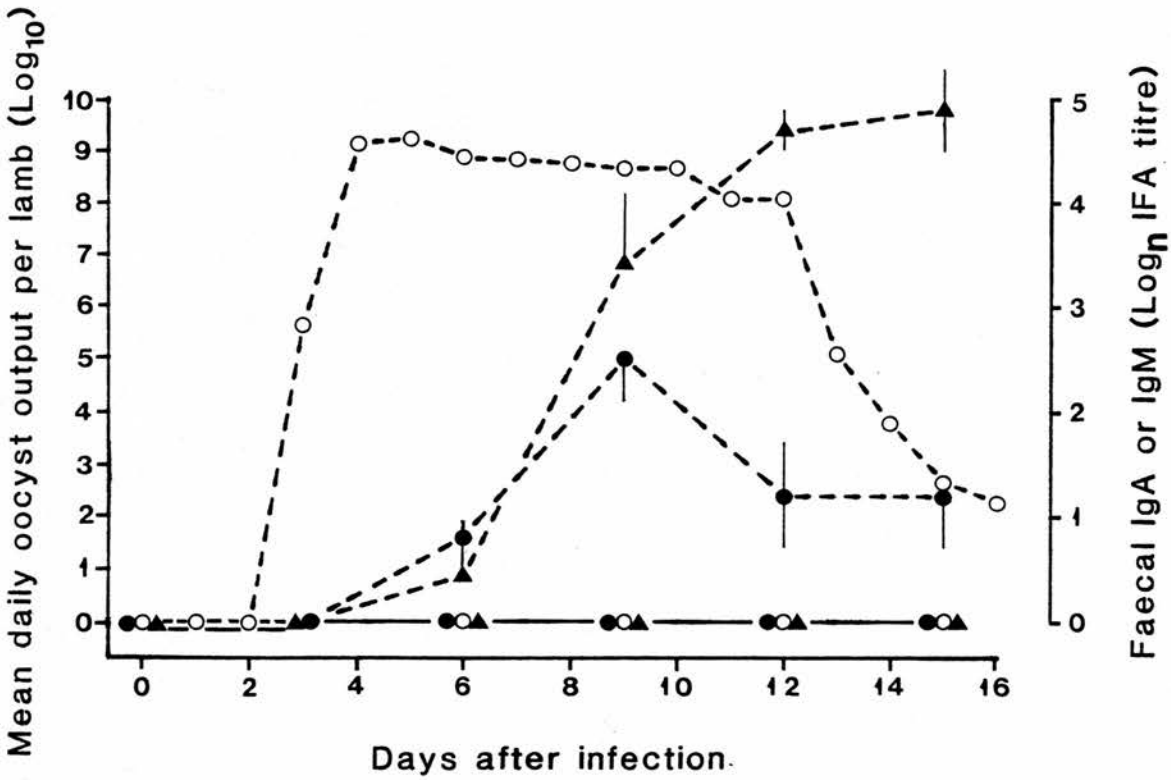

Mean daily oocyst output per lamb (log_{10})
Faecal IgA (Log_n IFA titre)
Days after infection
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28

Figure 4.4. Group mean oocyst excretion and faecal immunoglobulin titres expressed as $\log_{10}$ total oocysts/day/lamb (○) and $\log_n$ IFA titre (±SEM) (IgA, ▲; IgM, ●) respectively from gnotobiotic lambs infected (- - -) with 10^6 *Cryptosporidium* oocysts at 5 days of age and uninfected control (——) lambs. Values represent the mean of measurements from 6 principal and 2 control male lambs.


Mean daily oocyst output per lamb (Log10)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
Days after infection
Faecal IgA or IgM (Logn IFA titre)
5
4
3
2
1
0

IgA for gnotobiotic lambs but it was detected earlier, at 6 days after infection, and group mean titres tended to be higher than those of conventional lambs. Specific faecal IgM was detected only in gnotobiotic lambs and the highest group mean titre occurred at 9 days after infection. IgG was not detected in faecal extracts from gnotobiotic lambs.

Analysis of intestinal mucus

Specific IgA and IgM were detected in mucus extracts from gnotobiotic lambs killed 8 days after infection but only IgA was detected in mucus from lambs killed 16 days after infection (Figure 4.5a,b). Specific IgG was not detected at 8 or 16 days after infection. At 8 days after infection both IgM and IgA titres were relatively constant along the whole intestine. At 16 days after infection IgA titres tended to be higher in terminal ileum, caecum and colon.

Colonic mucus extract from a gnotobiotic lamb, 16 days after infection, was fractionated by gel filtration on a Bio-Gel A-1.5m column. Figure 4.6 shows the absorbance profile, elution volume and those fractions which were pooled for further analysis. The large absorbance peak at 450ml elution volume was not investigated as attention was focused on the initial excluded peak, containing mucus glycoprotein, and antibody associated with it. Under the same filtration conditions, a similar absorbance profile was found for extract prepared from the same weight of colonic mucus from an age-matched uninfected control lamb; the same fractions as those shown for the infected lamb were pooled for analysis.

Using purified sheep IgM, IgA and IgG in double diffusion with specific antisera, the lower limits of detection for these immunoglobulins were 80-100, 5-10 and 5-10 μg/ml respectively. Using the same antisera, the distribution of the immunoglobulin classes in pooled colonic mucus fractions from principal and

Figure 4.5a,b. Examination of soluble extracts, prepared from mucus collected from the intestinal tracts of gnotobiotic lambs, for specific antibody to *Cryptosporidium*. Principal lambs were infected at 5 days of age, killed at either 8 (Figure 4.5a) or 16 (Figure 4.5b) days after infection and compared with age-matched controls. Antibody was assayed with FITC-labelled pig antisheep IgM(●), IgA(▲) and IgG(■) conjugates on mucus from control (——) and infected (- - -) lambs. Intestinal segments 1-7 denote proximal to distal small intestine respectively; 8 is caecum; 9 is colon. Values represent the means (±SEM) of 4 principal and 2 control lambs (8 days after infection) and 6 principal and 4 control lambs (16 days after infection).

Figure 4.5a

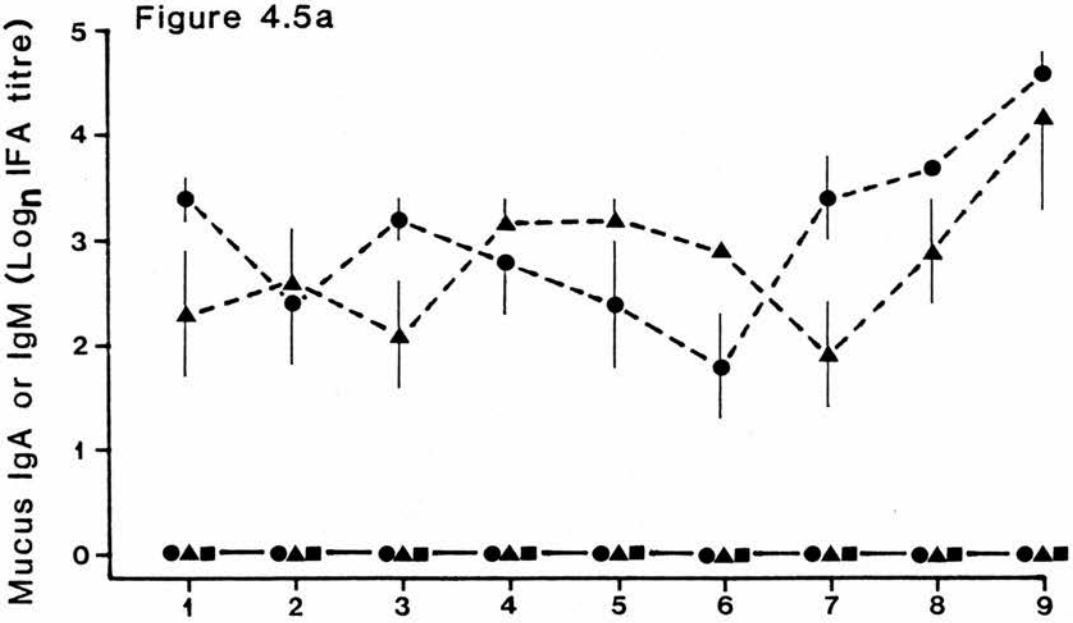
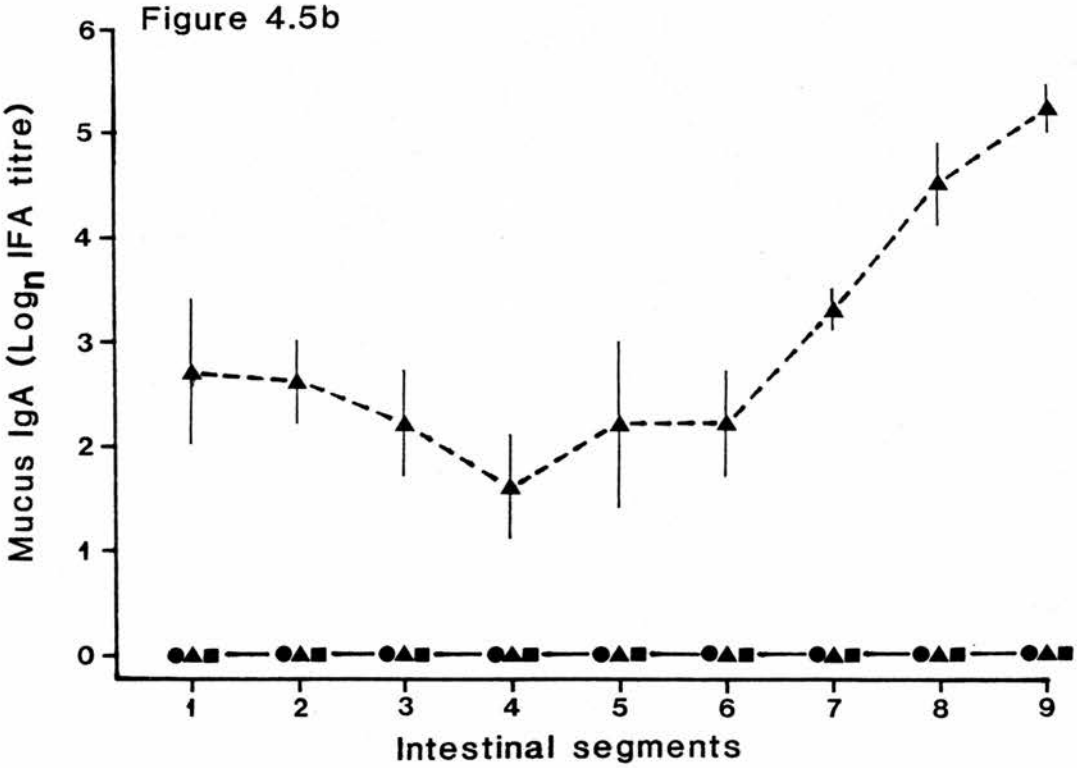


Figure 4.5b



(1–7 small intestine, 8 caecum, 9 colon)

Figure 4.6. Fractionation of soluble colonic mucus extract, from a *Cryptosporidium* infected lamb, by filtration through a Bio-Gel A-1.5m column.

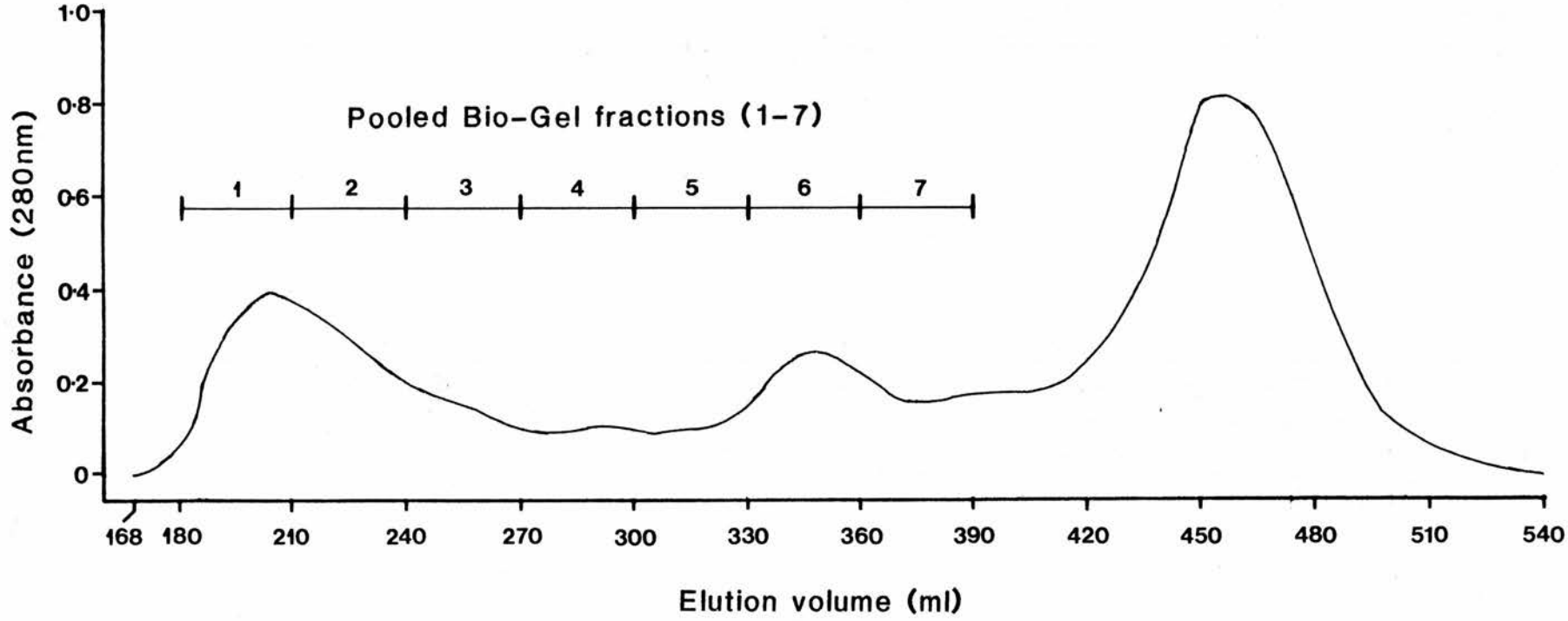
Column: 2.6cm diameter, bed height 90cm.

Fraction size: 6ml; pooled fractions (1-7, ⊢—⊣)

Flow rate: 18ml/hour of PBS (pH 7.1) containing 0.02% w/v sodium azide.

Sample: soluble extract prepared from 8 grams of crude colonic mucus collected from a gnotobiotic lamb 16 days after infection. Sample volume was 3ml.

After filtration, fraction pools 1-7 were concentrated to 1ml by vacuum dialysis against PBS.


Pooled Bio-Gel fractions (1-7)
1
2
3
4
5
6
7
Absorbance (280nm)
1·0
0·8
0·6
0·4
0·2
0
168
180
210
240
270
300
330
360
390
420
450
480
510
540
Elution volume (ml)

control lambs is shown in Figure 4.7a,b. IgG was not detected in pooled fractions examined from principal or control specimens. A weakly staining double band was detected in pooled principal fraction 3 using the IgM antisera. IgA was detected in principal fractions 2-7 but staining was most pronounced in fractions 3 and 4. An inner band was detected in fraction 6 using the IgA antisera which may have been free secretory component. IgA was detected only in pooled control fractions 3 and 4. IgM was not detected in control fractions. Fractions were further analysed by immunoelectrophoresis to determine the distribution of those proteins which formed precipitation lines with an anti-whole sheep serum. The pattern of precipitation arcs detected was similar for both principal and control mucus with most arcs detected on the side of the anode, in fractions 5, 6 and 7 (Figure 4.8a,b). There was little precipitation in the first four fractions.

After identical filtration and concentration procedures, mucus extracts prepared from 8 gram samples of principal and control crude mucus resulted in similar hexose fraction distributions (Figure 4.9). The largest quantity of mucus glycoprotein, as detected by analysis for hexose, was found in the second fraction pool for both principal and control specimens. The only specific antibody detected was IgA from fractions of principal mucus extract and this occurred after the hexose peak.

Section 2. Antibody response in rats

Kinetics

After a single infection at 4 days of age serum antibody to *Cryptosporidium* was first detected 5 days later and the group mean titre continued to rise until the conclusion of the experiment when the rats were 37 days of age. A similar serum antibody response was found up until 26 days of age in rats infected three times, after which a higher group mean titre was

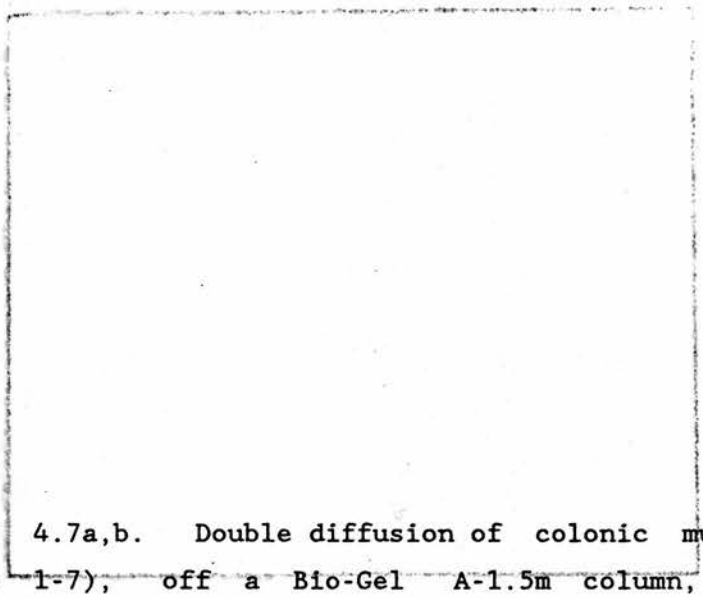


Figure 4.7a,b. Double diffusion of colonic mucus fractions (wells 1-7), off a Bio-Gel A-1.5m column, against pig antisheep IgM, IgA and IgG. Antisera were denoted as aIgM, aIgA and aIgG and were used neat. Well volumes were 10µl. The fractions shown in 4.7(a) and (b) were those described in Figure 4.6. They were derived from separate 8 gram samples of crude colonic mucus collected from a gnotobiotic principal lamb (16 days after *Cryptosporidium* infection) and an age-matched control.

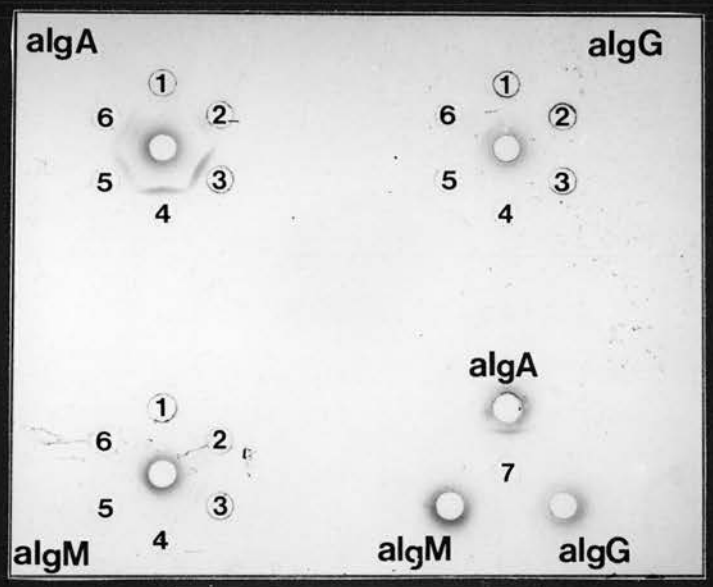


Figure 4.7a (Principal)

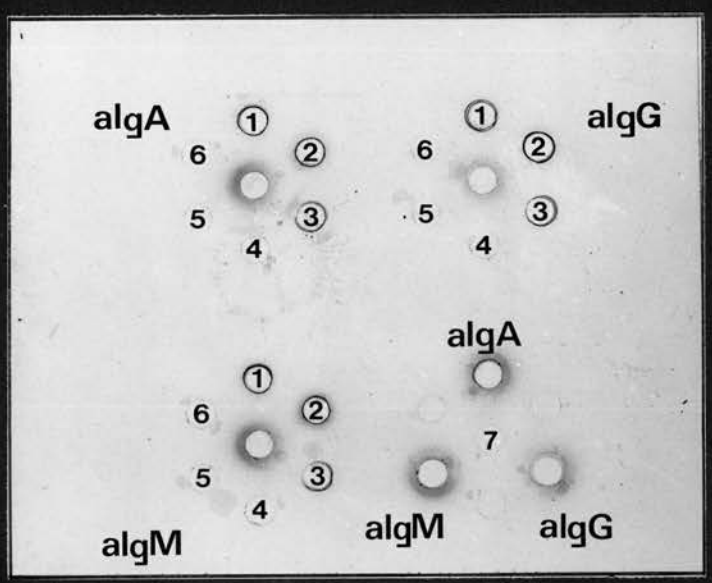


Figure 4.7b (Control)

Figure 4.8a,b. Immunoelectrophoresis of colonic mucus fractions (wells 1-7), off a Bio-Gel A-1.5m column, against pig anti-whole sheep serum. Troughs were filled with antiserum used neat. Well and trough volumes were 10µl and 100µl respectively. The fractions shown in 4.8(a) and 4.8(b) were those described in Figure 4.6. They were derived from separate 8 gram samples of crude colonic mucus collected from a gnotobiotic principal lamb (16 days after *Cryptosporidium* infection) and an age-matched control.

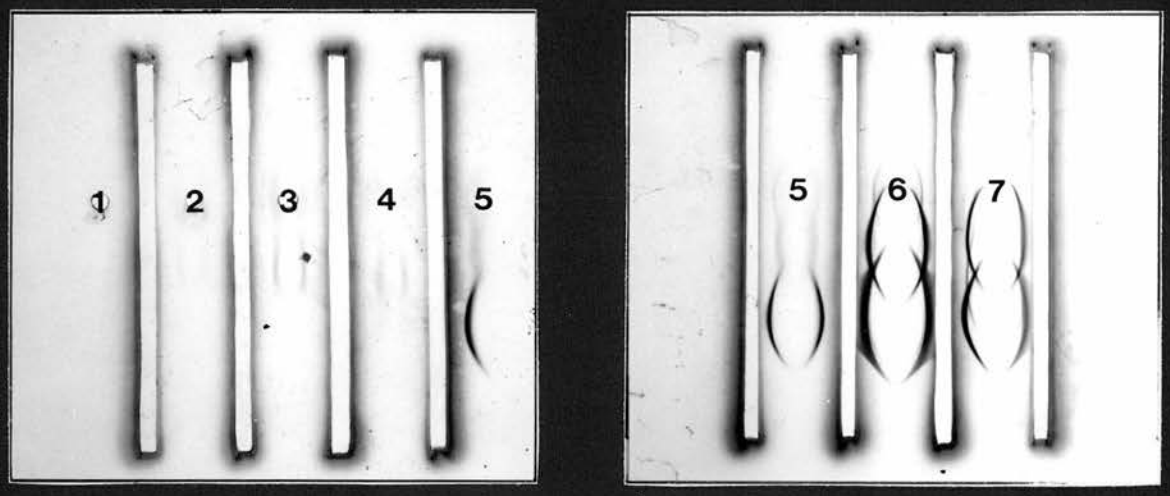


Figure 4.8a (Principal)

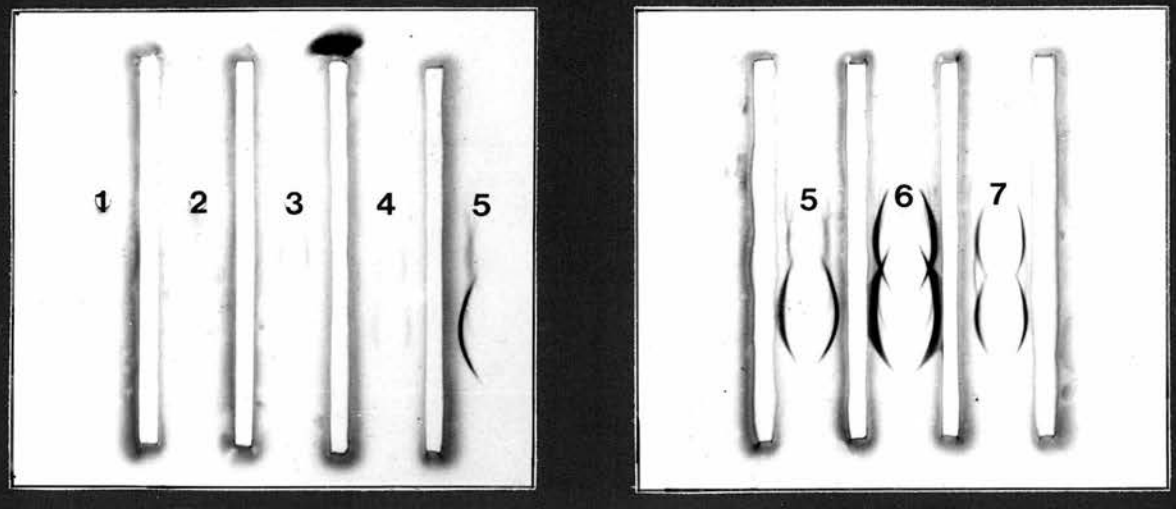


Figure 4.8b (Control)

Figure 4.9. Analysis of Bio-Gel fractions of soluble colonic mucus extracts, from a *Cryptosporidium* infected lamb(shaded) and an uninfected control lamb(unshaded).

Pooled fractions 1-7 correspond to those described in Figures 4.6, 4.7 and 4.8.

(a) The fraction range of total IgA (from Figure 4.7).

(b) The distribution of mucus glycoprotein in the fractions as determined by estimation of hexose (Dubois *et al*, 1956) using D-galactose as the standard (Chapter 2.10).

(c) Examination of fractions for specific antibody to *Cryptosporidium* using IFA (Chapter 2.9.3).

Figure 4.9a

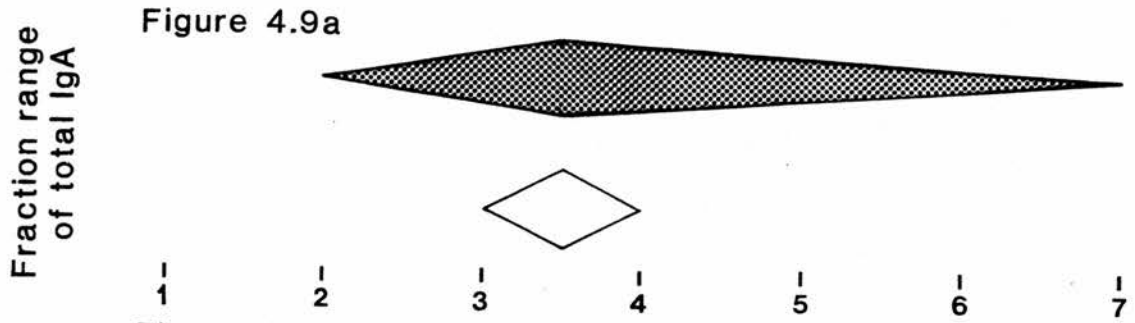


Figure 4.9b

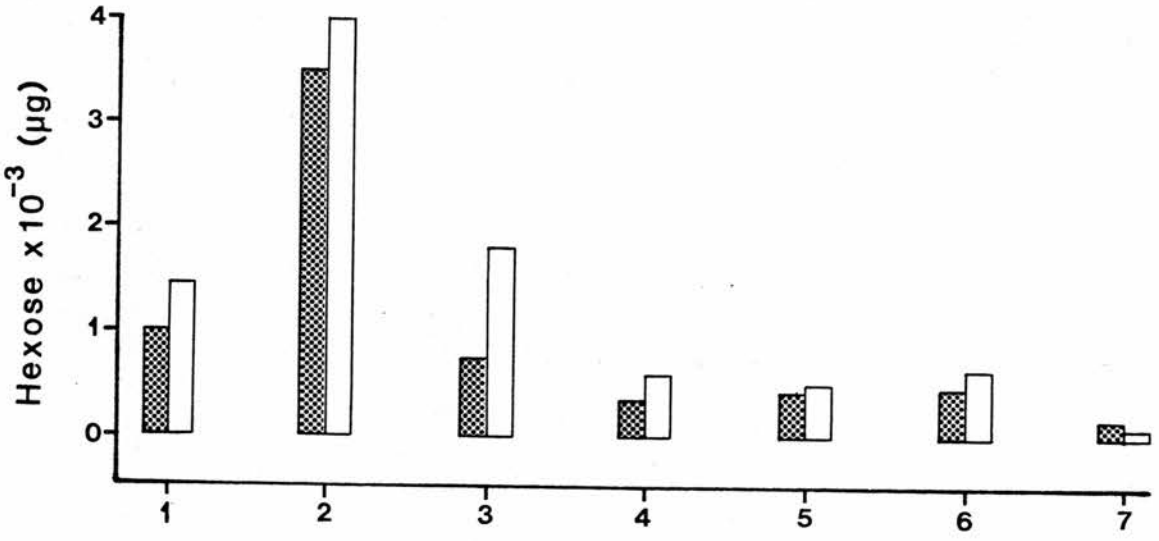


Figure 4.9c

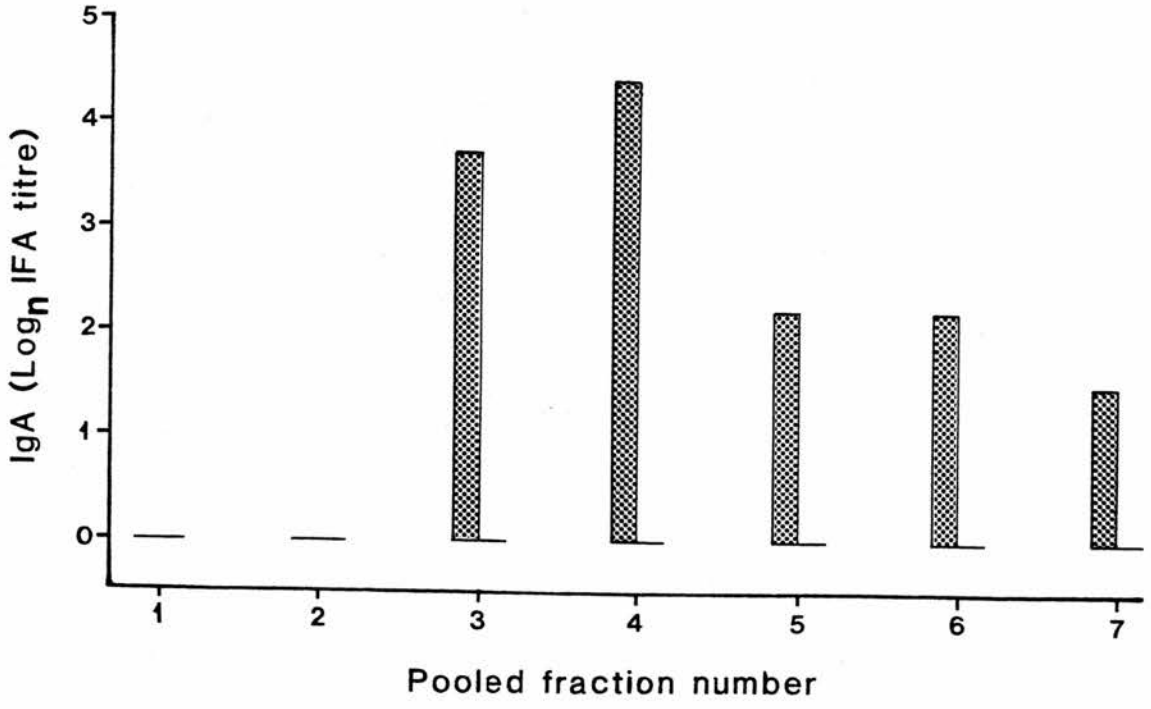


Figure 4.10a,b. Changes in total endogenous stage population density score and group mean specific serum antibody titre of rats infected with *Cryptosporidium*. Groups of Lister rats were infected once (▲ group 1), twice (● group 3) or three (■ group 2) times with 10^6 *Cryptosporidium* oocysts (cervine isolate) at the ages indicated (↓): (a) the total population density is shown for groups of rats infected twice and three times with *Cryptosporidium* (b) antibody assays were done with FITC - labelled rabbit anti-rat immunoglobulin conjugate on sera from control (□) and infected rats. Values represent the group mean $\log_n$ IFA titre (±SEM) from 6 rats killed at the ages indicated.

Figure 4.10a

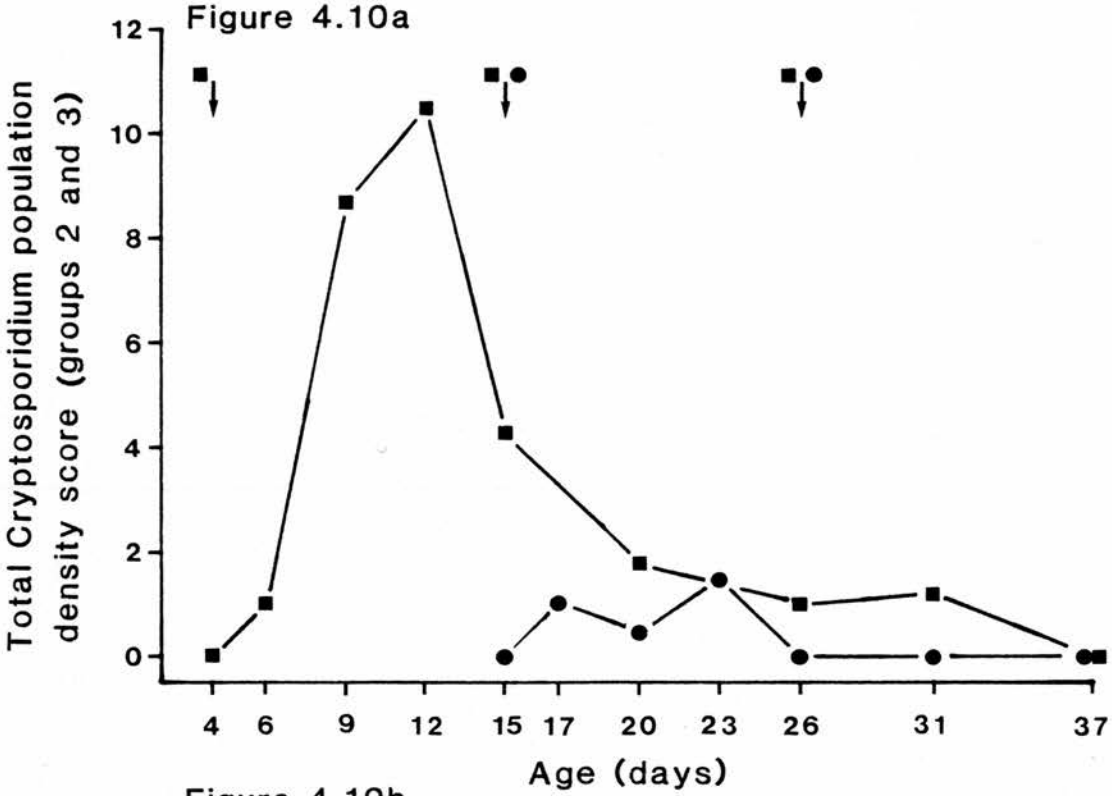
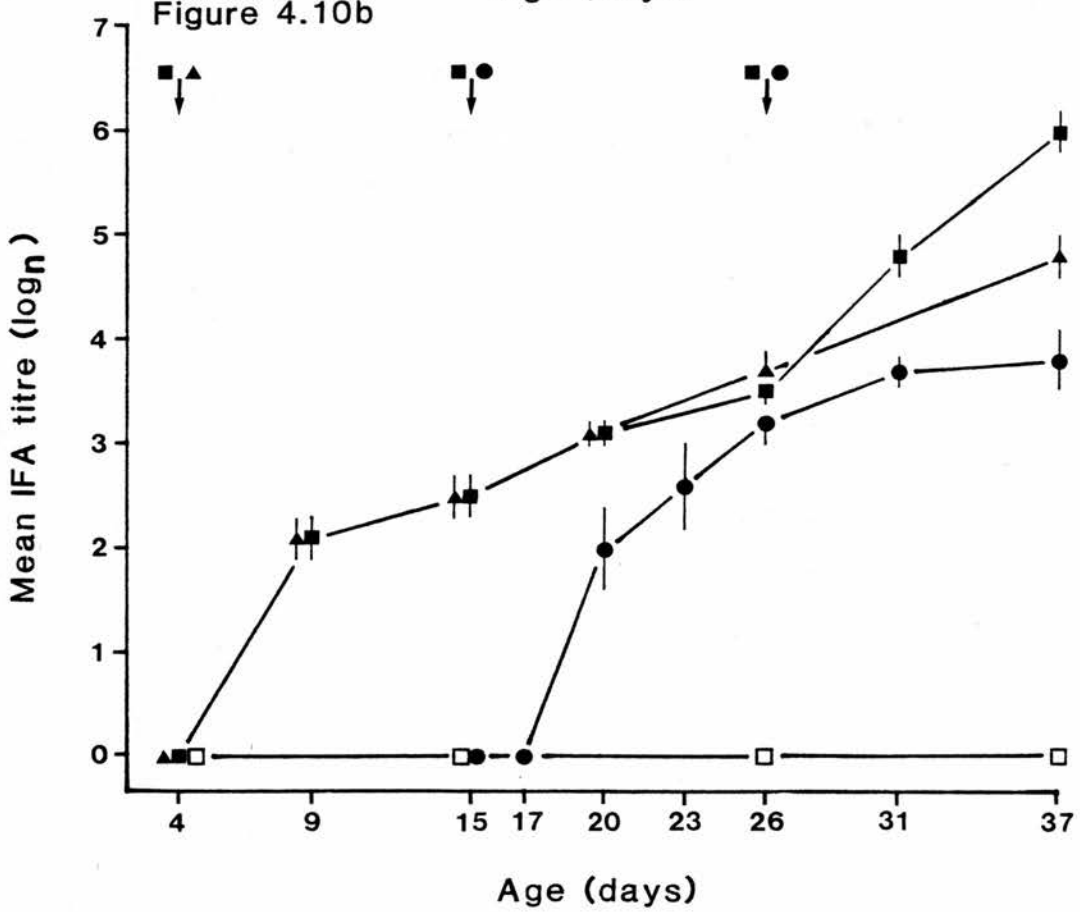


Figure 4.10b



detected at 37 days ($p<0.01$). The group mean serum titre of rats infected twice, initially at 15 and then at 26 days of age, showed a steady rise but at 37 days was lower than those rats infected once($p<0.05$) or three times($p<0.001$) (Figure 4.10a,b).

At 22 days after initial infection there were no significant differences between group mean serum titres; this was independent of whether rats had been infected once or twice and the age at which they were initially infected. However, eleven days after the first infection, rats infected at 15 days of age had a higher group mean serum titre($p<0.05$) than those infected at 4 days.

Discussion

This study of cryptosporidiosis provides the first detailed analysis of the time course of specific serum and copro-antibody responses and of the effects of reinfection. For lambs, any influence of passively acquired antibody on the progress of the infection and detection of endogenously produced specific antibody was eliminated by using colostrum-deprived animals.

In both lambs and rats specific serum antibodies were detected within one week of the inoculation of oocysts. In lambs, specific serum IgM was detected before IgG. The sequential appearance in serum, of IgM followed by IgG, has also been demonstrated with *Eimeria tenella* and *E. nieschulzi* infections in chickens and rats respectively (Rose, Peppard and Hobbs, 1984; Mockett and Rose, 1986).

Although the time course of individual immunoglobulin classes in lamb serum was not examined beyond 16 days after infection, total serum titres, detected by antisheep IgG (H+L), remained elevated until day 46 when the experiment ended. Since the half-life of sheep IgG is approximately 14 days (Smith *et al*, 1976), the persistent titre could be explained if there was

systemic dissemination of *Cryptosporidium* antigen from undetected residual infection. The methods used to examine faeces for oocysts (Chapter 2.3) were not sensitive enough to detect all oocysts shed. Furthermore, *Cryptosporidium* endogenous stages observed histologically in the intestines of normal young adult sheep (Angus *et al*, 1982a) and oocysts collected from faeces of adult sheep and cattle (Papadopoulou *et al*, 1988) indicate that a low level carrier status exists in the adult population.

Coproantibody specific for *Cryptosporidium* sporozoite antigens was detected in faecal extracts from infected lambs using FITC - conjugated antisheep alpha- and mu-chain specific, but not gamma-chain specific immunoglobulins. Most IgA in sheep intestinal secretions is produced by plasma cells in the lamina propria (Lascelles, Beh, Kerlin, Watson and Mukkur, 1985). This study showed that the rise in faecal IgA titres between days 8 and 16 coincided with the fall in oocyst output.

If the intestine's capacity to transport IgA is saturated, the excess overspills into the serum via intestinal lymph (Quin, Husband and Lascelles, 1975; Sheldrake, Husband, Watson and Cripps, 1984). Transport of either serum-derived or locally produced IgA into the intestinal lumen is dependent upon the number of IgA molecules competing for available secretory component molecules on the basal membrane of epithelial cells (Sheldrake, Husband, Watson, Barger and Boray, 1988). The peak specific serum IgA titre found at 11 days post infection may reflect intense synthesis of this antibody in the intestine at a time when insufficient secretory component was available to transport all the IgA produced.

Failure to detect IgM and IgG in faecal extracts from conventional lambs and IgG in that of gnotobiotic lambs may have been due to proteolytic degradation of these immunoglobulins. Similarly, analysis of intestinal washings from birds infected

with *E. tenella* (Davis, Parry and Porter, 1978) and rats infected with *E. nieschulzi* (Rose *et al*, 1984) have shown that IgA was the predominant immunoglobulin while IgG and IgM were barely detectable. However, in rotavirus and coronavirus infected calves, the sequence of appearance and time course of IgM and IgA in both serum and faecal specimens were similar to those observed in *Cryptosporidium* infected gnotobiotic lambs in this study (Saif, 1987).

IgA was the only specific isotype detected in intestinal mucus from gnotobiotic lambs killed 16 days after infection. Fractionation of colonic mucus by gel filtration showed that IgA from the infected lamb had a greater molecular weight range than that of the control. The greater concentration of IgA likely to have been present in principal mucus extract and possible degradation to monomeric forms would have resulted in greater spread of IgA among fractions. Although double diffusion is a qualitative procedure, the intensity of the staining pattern indicated that most of the IgA was present in fractions 3 and 4 from both principal and control lambs. Any non-covalent association between secretory IgA and mucus (Clamp, 1977) may have been altered by homogenization procedures (Chapter 2.10) aimed at solubilizing mucus glycoprotein. This may have influenced the distribution of IgA found in the present experiment. Bio-Gel fractions containing the bulk of the mucus glycoprotein (as detected by hexose analysis) showed little reaction with anti-whole sheep serum on immunoelectrophoresis. Most of the IgA and other serum-derived proteins were detected in fractions eluting after the excluded peak of mucus glycoprotein.

In rats, the greatest proliferation of endogenous stages occurred after a primary infection given at 4 days of age. However, at eleven days after a primary infection, rats infected at 15 days had higher serum titres than those infected at 4 days. This may have been due to immature immune response in the

younger rats, greater emphasis on production of secretory antibody or immunosuppressive effects of the parasite. While many species of protozoa have been shown to have immunosuppressive effects upon the host (Wakelin, 1984), this has not been demonstrated with *Cryptosporidium*. Measurement of secretory antibody was not attempted in this study due to technical difficulties in collecting specimens from the intestines of young rats. In young rats Peyer's patches begin to resemble those of the adult at approximately 12 days of age (Wilders *et al*, 1983) and they have been shown to be important in regulating the intestinal immune response in this species (Enders *et al*, 1988). Hence, rats infected at 4 days of age may not have responded as well to *Cryptosporidium* antigen.

There was no convincing evidence of a secondary antibody response in the rat model. Specific antibody responses after the second and third inocula were elevated but did not appear accelerated. Furthermore, an elevated response after reinfection was difficult to interpret since specific antibody titre continued to rise over the observation period even in those rats infected only once, at 4 days of age.

CHAPTER 5. Analysis of the specificity of antibodies produced by lambs and rats infected with *Cryptosporidium*

Introduction

In Chapter 4 the kinetics of the antibody response to *Cryptosporidium* infection was investigated in relation to oocyst shedding and endogenous stage population density in the lamb and Lister rat respectively. In the lamb it was shown that the rise in IgA and IgM titres in faecal extracts coincided with the fall in oocyst output. The rat studies showed that *Cryptosporidium* endogenous stage population density decreased in association with both rising serum antibody titres (Chapter 4, Section 2) and an age-related resistance to infection (Chapter 3, Experiment 3.2).

This chapter extends the investigation of antibodies in cryptosporidiosis by analysing their specificity on immunoblots of oocysts and merozoites. The aims were: to compare the specificities of serum and copro-antibodies from lambs, to investigate antigens common to both oocyst and merozoite, and to determine whether the specificity of convalescent rat sera is influenced by the age of the animal at initial infection and subsequent challenge.

Experimental design

Serum and coproantibody were obtained from colostrum deprived conventional and gnotobiotic lambs used in Experiment 3.5 (Chapter 3). Principal lambs were infected orally with 10^6 *Cryptosporidium* oocysts (cervine isolate) at 5 days of age and killed 16 days after infection.

Serum specimens were obtained from Lister rats allocated to either control or to one of three principal groups, described in Experiment 3.2 (Chapter 3). Rats were infected either once (at 4

days of age, group 1), three times (at 4, 15 and 26 days of age, group 2) or twice (at 15 and 26 days of age, group 3) with 10^6 *Cryptosporidium* oocysts (cervine isolate) given orally on each occasion.

The extraction of coproantibody from lamb faeces and the production of hyperimmune sera raised against *Cryptosporidium* are described in Chapter 2, Sections 2.6.1 and 2.9.1. respectively.

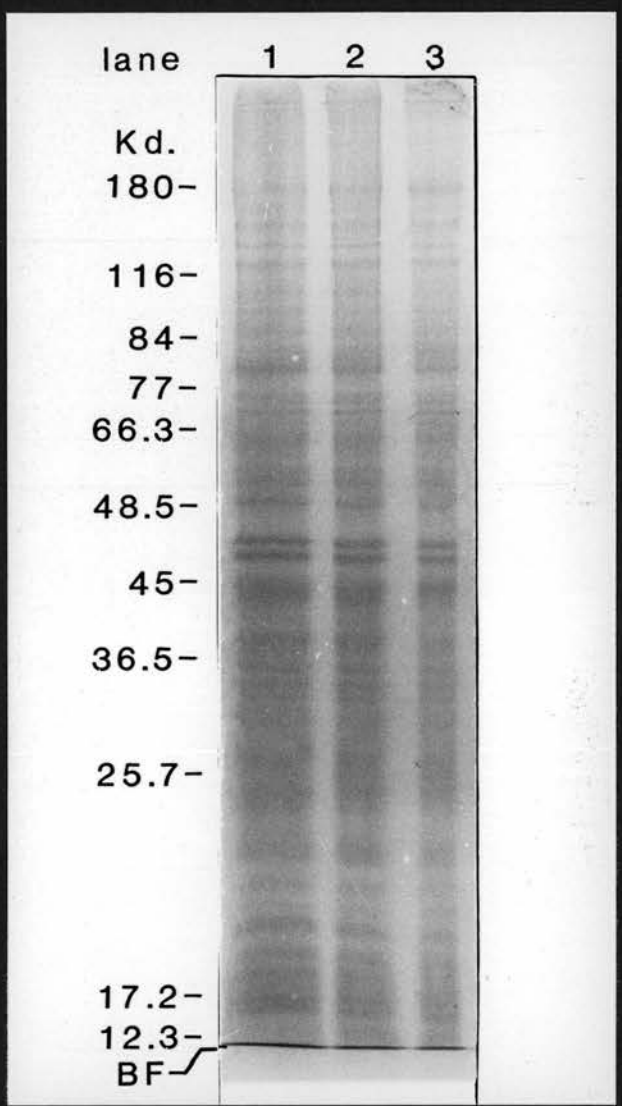
The preparation of *Cryptosporidium* oocyst and merozoite antigens from the cervine isolate and the method of their analysis by SDS-PAGE and immunoblotting procedures are described in Chapter 2, Sections 2.9.7. and 2.9.8. Numbers shown in the left margin of polyacrylamide gel and immunoblot illustrations are molecular weight markers in kilo-daltons (Kd.).

Results

Section 1. SDS-PAGE analysis of *Cryptosporidium* oocyst antigen

Oocyst antigen was derived by denaturation and reduction of proteins from both oocyst walls and sporozoites. A complex pattern of polypeptides was revealed when the gel was stained with Coomassie blue (Figure 5.1). These polypeptides spanned a molecular weight range from above 180Kd. to below 12.3Kd. No additional polypeptides were detected at track loadings of antigen above that equivalent to 10^7 oocysts. This loading (lane 2, Figure 5.1) was used in all subsequent SDS-PAGE gels intended for immunoblotting.

Figure 5.1. SDS-PAGE of oocyst antigens derived from proteins contained in the oocyst wall and sporozoites. Well loadings in terms of equivalent oocyst numbers were: lane 1, $2x10^7$; lane 2, 10^7; lane 3, $0.5x10^7$. Electrophoresed proteins were stained with Coomassie blue. Molecular weight markers, in kilo-daltons(Kd.), are shown in the left hand margin. BF, buffer front.


lane
1
2
3
Kd.
180-
116-
84-
77-
66.3-
48.5-
45-
36.5-
25.7-
17.2-
12.3-
BF

Section 2. Immunoblot analysis of *Cryptosporidium* oocyst antigen

Hyperimmune rabbit sera

Immunoblot analysis of oocyst antigen (cervine isolate), with hyperimmune rabbit sera raised against bovine, cervine or equine isolates of *Cryptosporidium* (Table 2.2a), is shown in Figure 5.2. All except one (arrowed) of the antigens detected by hyperimmune serum raised against the cervine isolate had bands of identity with the other two hyperimmune sera. A double band of antigen at approximately 180Kd. (I), a single band at 23Kd. (V) and a broad band between 12.3 and 17.2Kd. (VI) are three of six reference point antigens to be described in this study (Figure 5.2). Other reference point antigens were not marked in this figure because they were not clearly defined among the numerous bands recognized by hyperimmune serum. Hyperimmune serum raised against the equine isolate showed a more intense reactivity with antigen V, compared to bovine and cervine hyperimmune sera. Between antigens V and VI there were no clearly recognized bands.

Sera and faecal extracts from experimental lambs

The oocyst antigens detected by antibodies in sera obtained at weekly intervals from one *Cryptosporidium* infected conventional lamb are shown in Figure 5.3. Bound antibodies were detected with anti-sheep $F(ab)_2$ specific immunoglobulin. Most of the antigens recognized throughout this period had molecular weights greater than 66Kd. A double band of antigen with molecular weight corresponding to I was a consistent feature in this region. Two antigens with molecular weights corresponding to V and VI were also consistent features on immunoblots of convalescent sera. Sera from a second infected conventional lamb gave similar results, although at one week post infection antigen V and others with molecular weights greater than 84Kd. were recognized.

Figure 5.2. Immunoblots of *Cryptosporidium* oocyst antigens developed with serum from normal and immunized rabbits.

Antigen derived from 10^7 oocysts was applied per lane. All lanes were developed using pig antisera to rabbit immunoglobulin (Table 2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, normal rabbit serum; hyperimmune rabbit sera raised against bovine(lanes 3 and 4), cervine(lanes 5 and 6) and equine(lanes 7 and 8) *Cryptosporidium* isolates. Reciprocal serum dilutions for each lane are shown at the bottom of the figure.

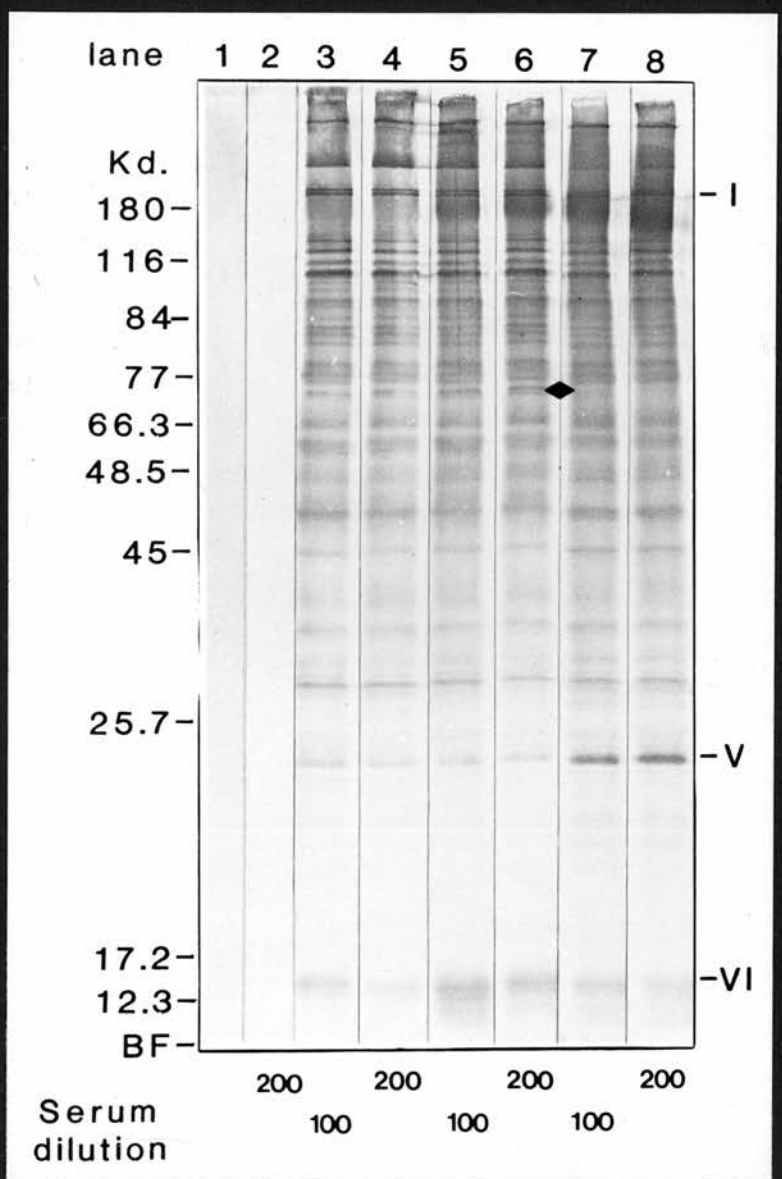
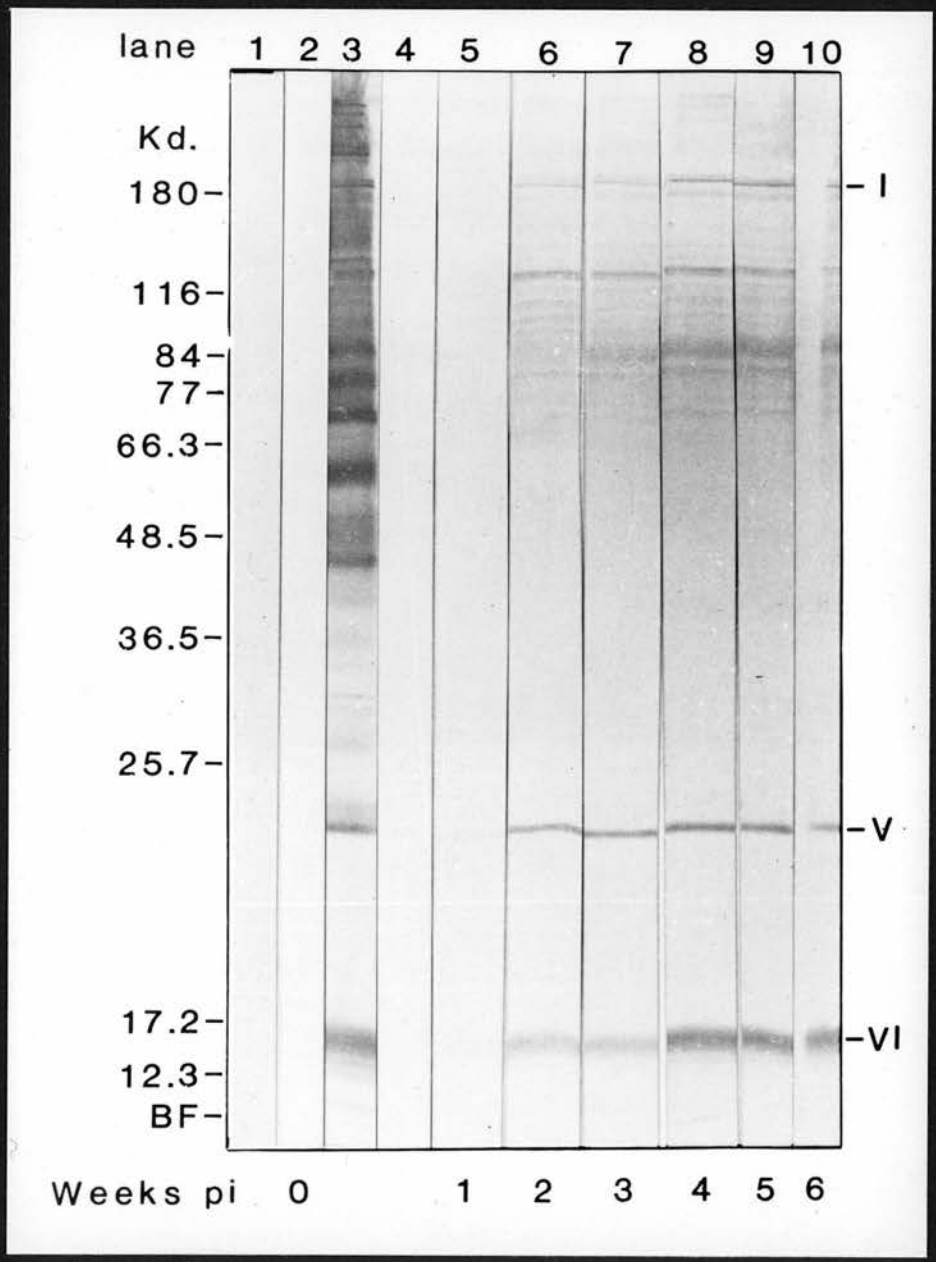

lane 1 2 3 4 5 6 7 8
Kd.
180
116
84
77
66.3
48.5
45
25.7
17.2
12.3
BF
I
V
VI
200 200 200 200
Serum dilution
100 100 100

Figure 5.3. The specificity of antibodies in conventional lamb sera for *Cryptosporidium* oocyst antigens at weekly intervals after infection.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using a pig anti-sheep $F(ab)_2$ conjugate (Table 2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, pre-inoculation serum; lane 3, 1:80 dilution of hyperimmune lamb serum (Table2.2b); lane 4, uninfected control lamb serum at 4 weeks of age; lanes 5-10, serial samples from an infected lamb bled at 1-6 weeks post-infection(pi) respectively. Control and post-infection sera were used at a dilution of 1:10.


lane 1 2 3 4 5 6 7 8 9 10
Kd.
180-
116-
84-
77-
66.3-
48.5-
36.5-
25.7-
17.2-
12.3-
BF-
I
V
VI
Weeks pi 0 1 2 3 4 5 6

The reaction of hyperimmune lamb serum (Table 2.2b) is shown in lane 3 of Figure 5.3 with antigens I, V, VI and many more being detected. Convalescent lamb sera, in contrast to hyperimmune rabbit and lamb sera, did not react with antigens between the 66Kd. marker and antigen V. Neither the convalescent nor hyperimmune lamb sera clearly recognized any antigens between V and VI (Figures 5.2 and 5.3).

Conjugate specific for mu-chain was used to examine sera from gnotobiotic lambs 10 days after infection with *Cryptosporidium* (Figure 5.4). This analysis revealed a greater range of antigens compared to that described above for conventional lambs. Three prominent single bands, labelled II, III and IV, were distinguished in immunoblots from most of these lambs and had approximate molecular weights of 93, 67 and 47Kd. respectively. Antigens I, II, III and IV were separated by a ladder-like series of minor bands.

Sera, obtained from these gnotobiotic lambs 15 days post infection were examined for IgG antibodies (Figure 5.5). Antigens with molecular weights corresponding to I, V and VI were detected and in addition, four antigens (arrowed) were prominent between the 180 and 116Kd. molecular weight markers.

To determine the time course and specificity of coproantibodies for *Cryptosporidium* oocyst antigens, immunoblots were made for each of three conventional lambs using faecal extracts obtained 0, 3, 7, 10, 16, 23 and 28 days after infection and developed with anti$F(ab)_2$. No reaction was detected at 3 days after infection, but faint, variable recognition of antigens with molecular weights greater than 84Kd. occurred from 10 days onwards. All three lambs reacted strongly with antigens V and VI on days 10 and 16, but this reaction faded variably between days 23 and 28. A typical example is shown in Figure 5.6.

Figure 5.4. The specificity of gnotobiotic lamb serum IgM for *Cryptosporidium* oocyst antigens 10 days after infection.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using pig antisera specific for sheep IgM (Table 2.3). Antigens: I (180Kd., double band); II (93Kd.); III (67Kd.); IV (47Kd.); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, age-matched uninfected control serum; lanes 3-8, sera from 6 different lambs at 10 days after infection. The same infected lambs are represented in correspondingly labelled lanes in Figures 5.5 and 5.8. Sera were used at a 1:10 dilution.

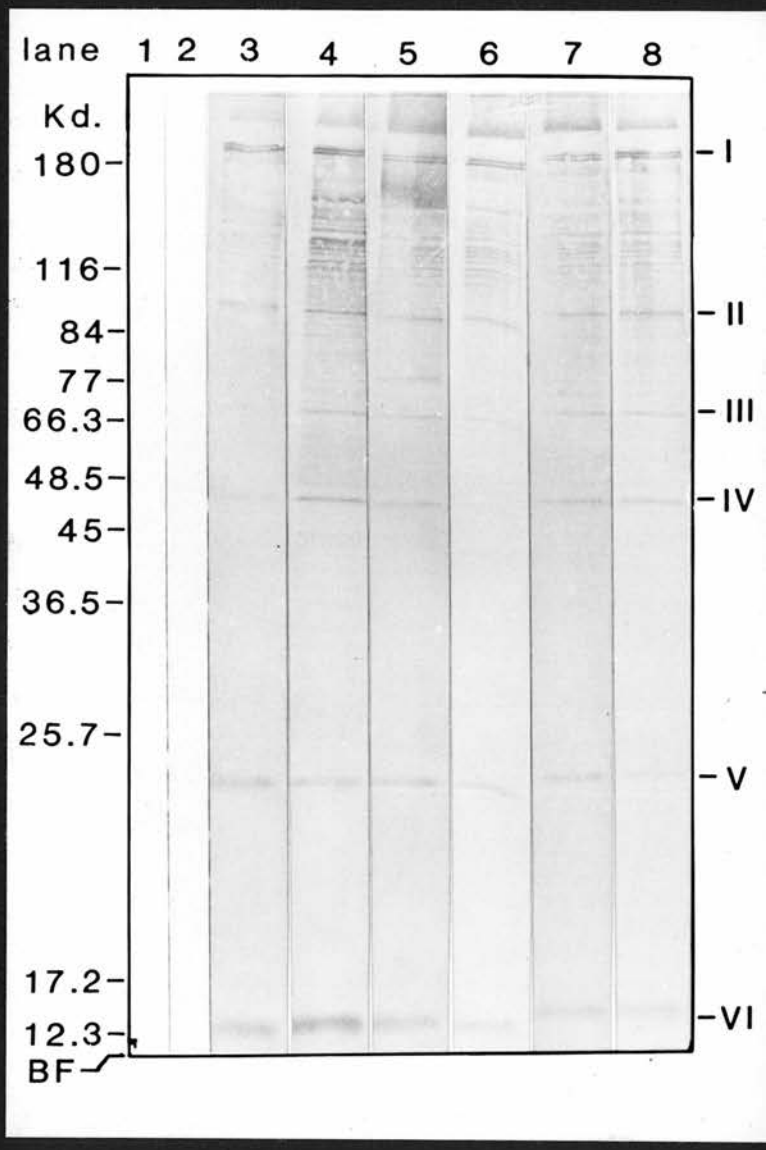

lane 1 2 3 4 5 6 7 8
Kd.
180
116
84
77
66.3
48.5
45
36.5
25.7
17.2
12.3
BF
I
II
III
IV
V
VI

Figure 5.5. The specificity of gnotobiotic lamb serum IgG for *Cryptosporidium* oocyst antigens 15 days after infection.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using a pig antisera specific for sheep IgG (Table 2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, age-matched uninfected control serum; lanes 3-8, sera from 6 different lambs at 15 days after infection. The same infected lambs are represented in correspondingly labelled lanes of Figures 5.4 and 5.8. Sera were used at a 1:10 dilution.

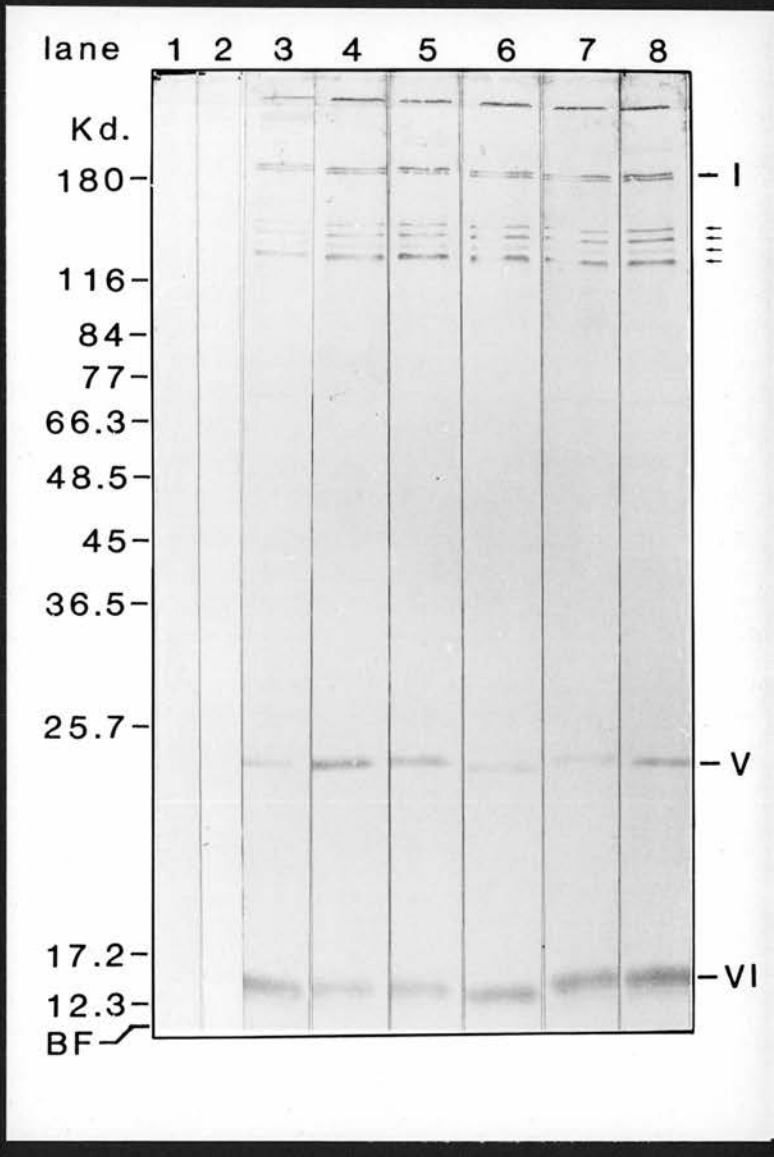
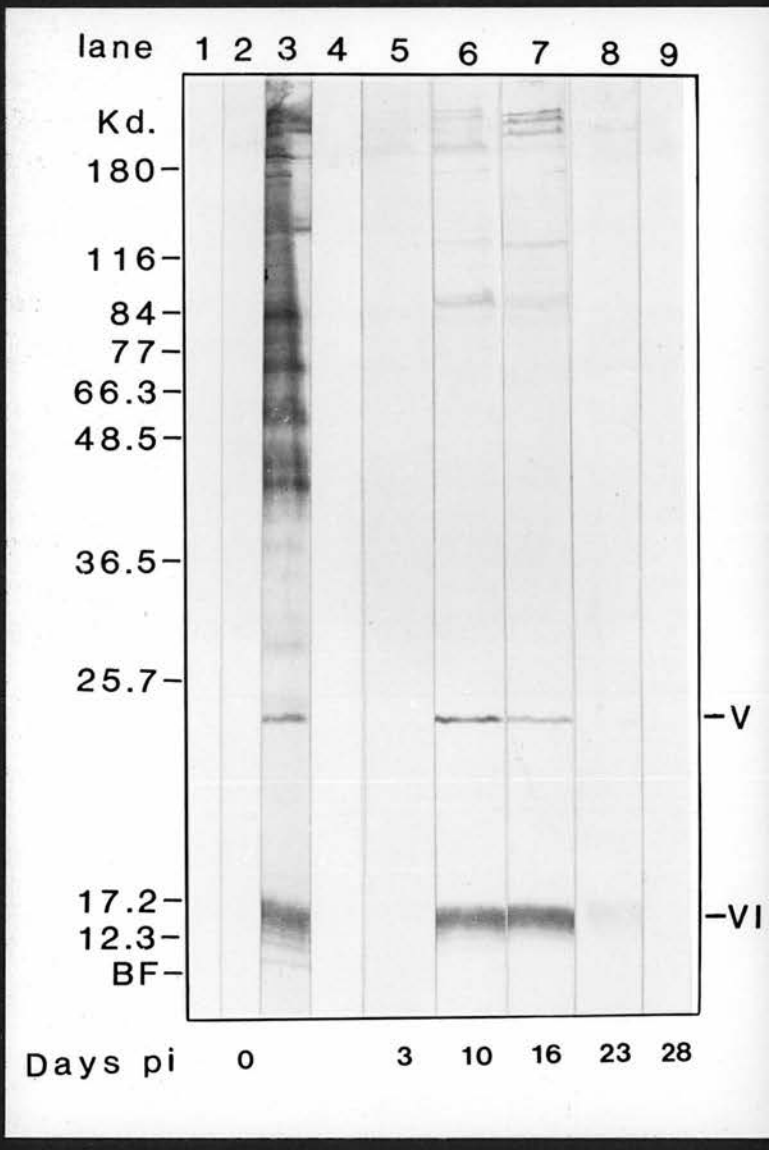

lane 1 2 3 4 5 6 7 8
Kd.
180
116
84
77
66.3
48.5
45
36.5
25.7
17.2
12.3
BF
I
V
VI

Figure 5.6. The specificity of coproantibodies from a conventional lamb for *Cryptosporidium* oocyst antigens after infection.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using pig anti-sheep $F(ab)_2$ conjugate (Table 2.3). Antigens: V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, pre-inoculation faecal extract; lane 3, 1:80 dilution of hyperimmune lamb serum (Table 2.2b); lane 4, faecal extract from an uninfected control lamb at 4 weeks of age; lanes 5-9, faecal extracts from one infected lamb at 3, 10, 16, 23, and 28 days post-infection(pi) respectively. Faecal extracts were used at a dilution of 1:10.


lane 1 2 3 4 5 6 7 8 9
Kd.
180
116
84
77
66.3
48.5
36.5
25.7
17.2
12.3
BF
V
VI
Days pi 0 3 10 16 23 28

For further investigation of antigens recognized by IgA, immunoblots were initially performed using conjugate specific for alpha-chain and coproantibodies from *Cryptosporidium* infected conventional lambs. This study was repeated using coproantibody from the same gnotobiotic lambs described in Figure 5.4. The results of IgA coproantibody analyses are shown in Figures 5.7 (conventional lambs) and 5.8 (gnotobiotic lambs). Antigens with molecular weights corresponding to I, V and VI were detected by IgA in faecal extracts from conventional lambs at 16 and 23 days and in gnotobiotic lambs at 15 days after infection. The range of other antigens recognized varied between immunoblots. As for immunoblots performed with serum, when antigens I, II, III and IV were detected, they were separated by a ladder-like series of minor bands. Antigens II, III and IV, if present, were not as clearly defined in IgA immunoblots from conventional lambs. Immunoblot analysis of coproantibody from all lambs in this study did not show any clearly recognized antigen between V and VI.

Sera from experimental rats

The serum antibody titres of Lister rats, during the course of single and multiple infections with *Cryptosporidium*, are described in Chapter 4. These data, together with the times (labelled A-G) after infection at which pooled rat sera were analyzed by immunoblotting, are shown in Figure 5.9. The results of immunoblotting analysis at these times are shown in the correspondingly labelled lanes of Figures 5.10 and 5.11.

Eleven days after rats were infected for the first time, those initially infected at 15 days of age (lane D) showed a stronger reactivity compared to those infected at 4 days (lane A, Figure 5.10). Antigens corresponding to I and V described above for lambs and others with molecular weights greater than 77Kd. were more clearly seen on immunoblots from the older group of rats. The reactivity against these antigens was strongest in rats 26

Figure 5.7. The specificity of IgA in faecal extracts from conventional lambs for *Cryptosporidium* oocyst antigens at 16 or 23 days after infection.

Antigen derived from 10^7 oocysts was applied to each lane. Lane 4 was developed using pig anti-sheep $F(ab)_2$ conjugate, while all other lanes were developed using a pig antisera specific for sheep IgA (Table 2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, IgA conjugate control; lane 2, faecal extract from an uninfected control lamb at 4 weeks of age; lanes 3 and 4, 1:80 dilution of hyperimmune lamb serum (Table 2.2b); lanes 5, 6, 8 and 10 and lanes 7 and 9, faecal extracts from different lambs at 16 and 23 days post-infection(pi) respectively. Faecal extracts were used at a dilution of 1:10.

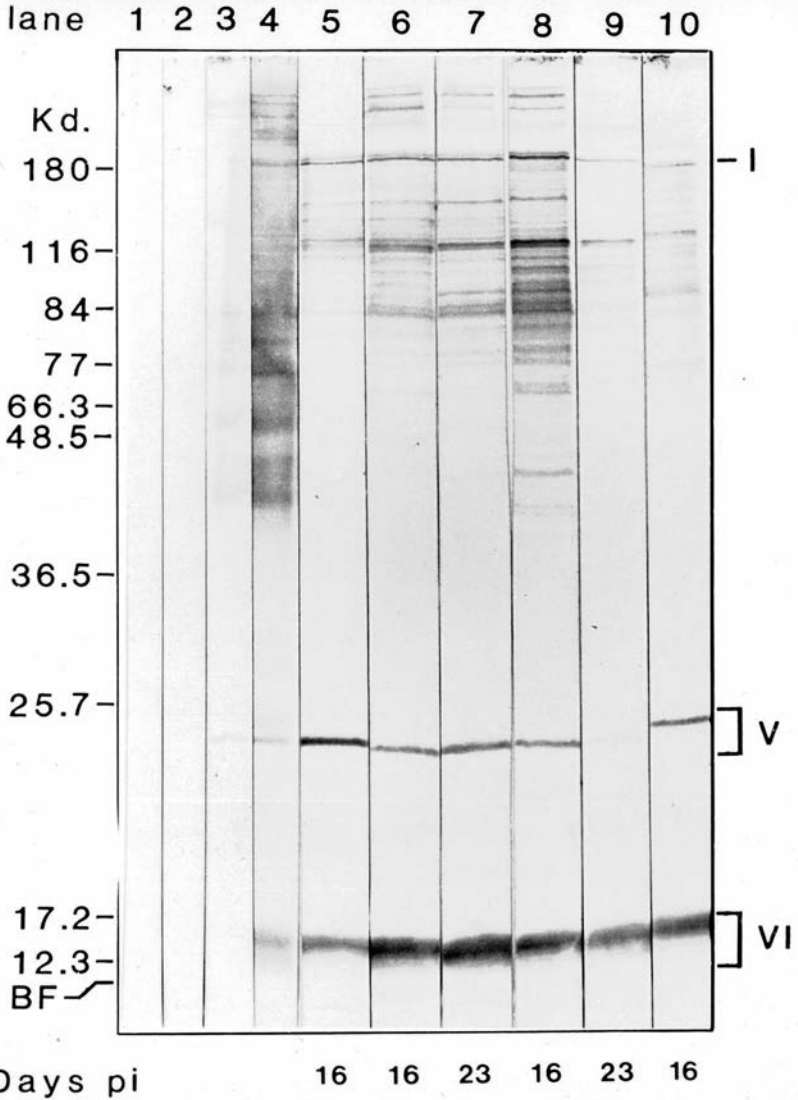

lane 1 2 3 4 5 6 7 8 9 10
Kd.
180
116
84
77
66.3
48.5
36.5
25.7
17.2
12.3
BF
I
V
VI
Days pi
16 16 23 16 23 16

Figure 5.8. The specificity of IgA in faecal extracts from gnotobiotic lambs for *Cryptosporidium* oocyst antigens 15 days after infection.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using pig antisera specific for sheep IgA (Table 2.3). Antigens: I (180Kd., double band); II (93Kd.); III (67Kd.); IV (47Kd.); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lane 2, faecal extract from an age-matched uninfected control lamb; lanes 3-8, faecal extracts from different infected lambs at 15 days after infection. Faecal extracts were used at a 1:10 dilution. The same infected lambs are represented in correspondingly labelled lanes in Figures 5.4 and 5.5.

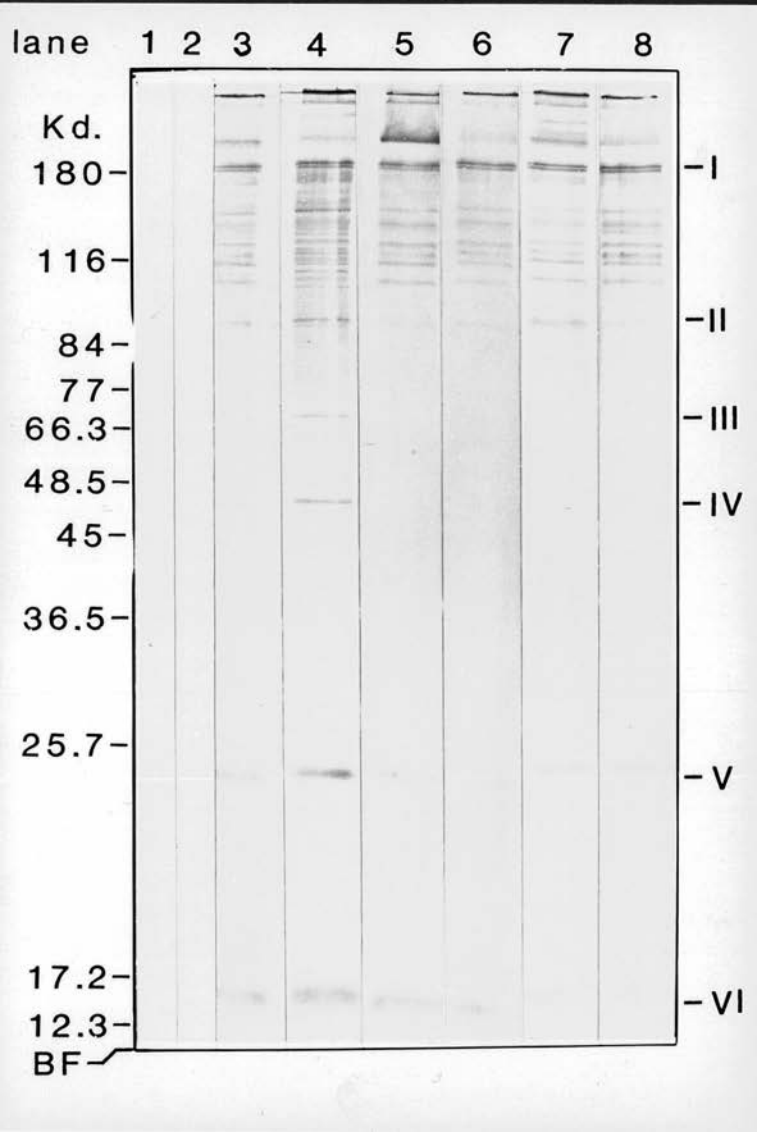

lane 1 2 3 4 5 6 7 8
Kd.
180
116
84
77
66.3
48.5
45
36.5
25.7
17.2
12.3
BF
I
II
III
IV
V
VI

Figure 5.9. The serum antibody response of rats infected with *Cryptosporidium* and the times of sampling for analysis of their specificity for oocyst antigens.

Groups of Lister rats were infected once (▲), twice (●), or three (■) times with 10^6 *Cryptosporidium* oocysts (cervine isolate) at the ages indicated (↓). Antibody assays were done with FITC-labelled rabbit anti-rat immunoglobulin (Table 2.3) on sera from control (□) and infected rats. Values represent the group mean $\log_n$ IFA titre from 6 rats killed at the ages indicated.

The specificity of antibody in pooled sera at times A-G was investigated using immunoblotting procedures. The results are shown in the correspondingly labelled lanes in Figures 5.10 and 5.11.

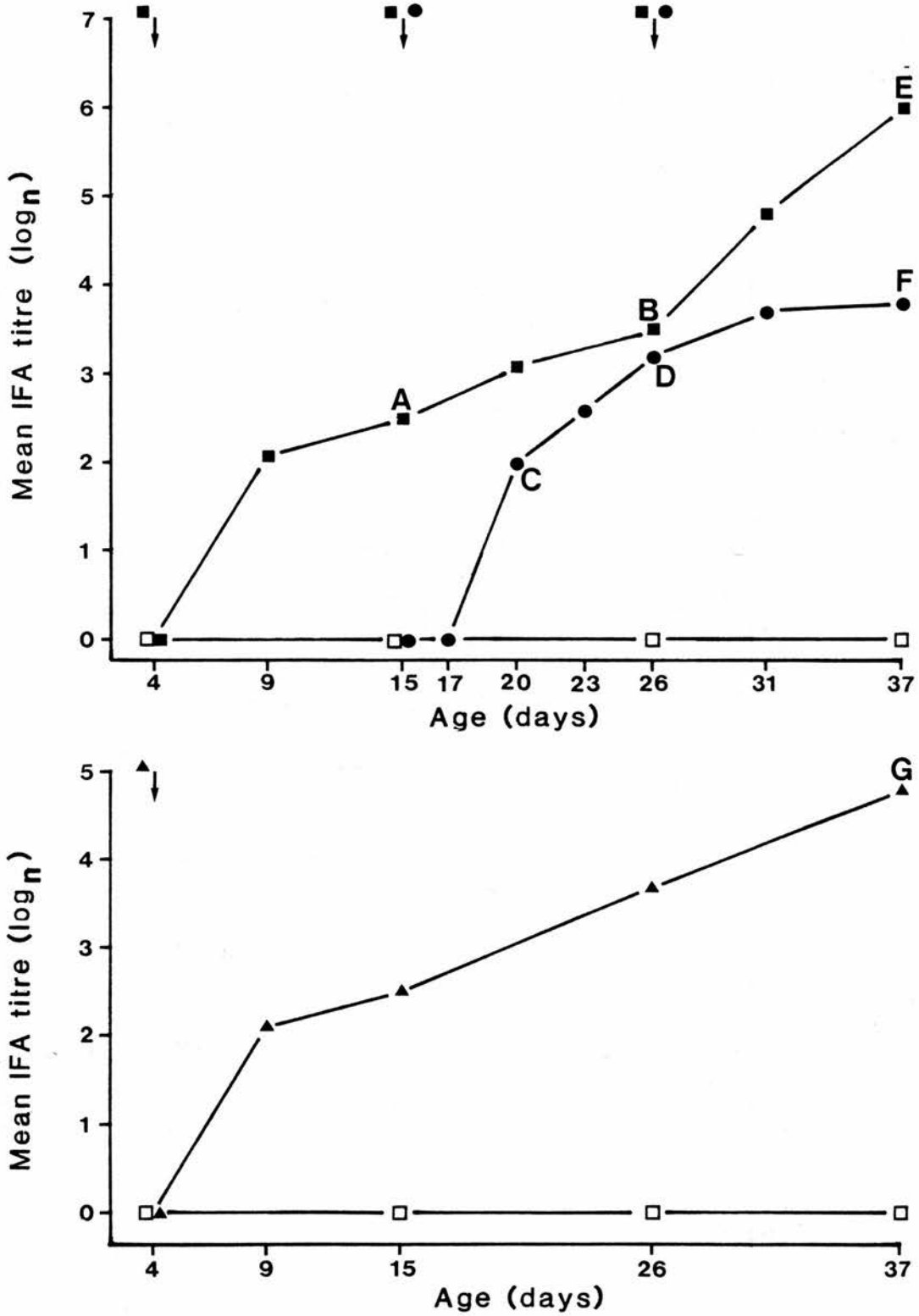
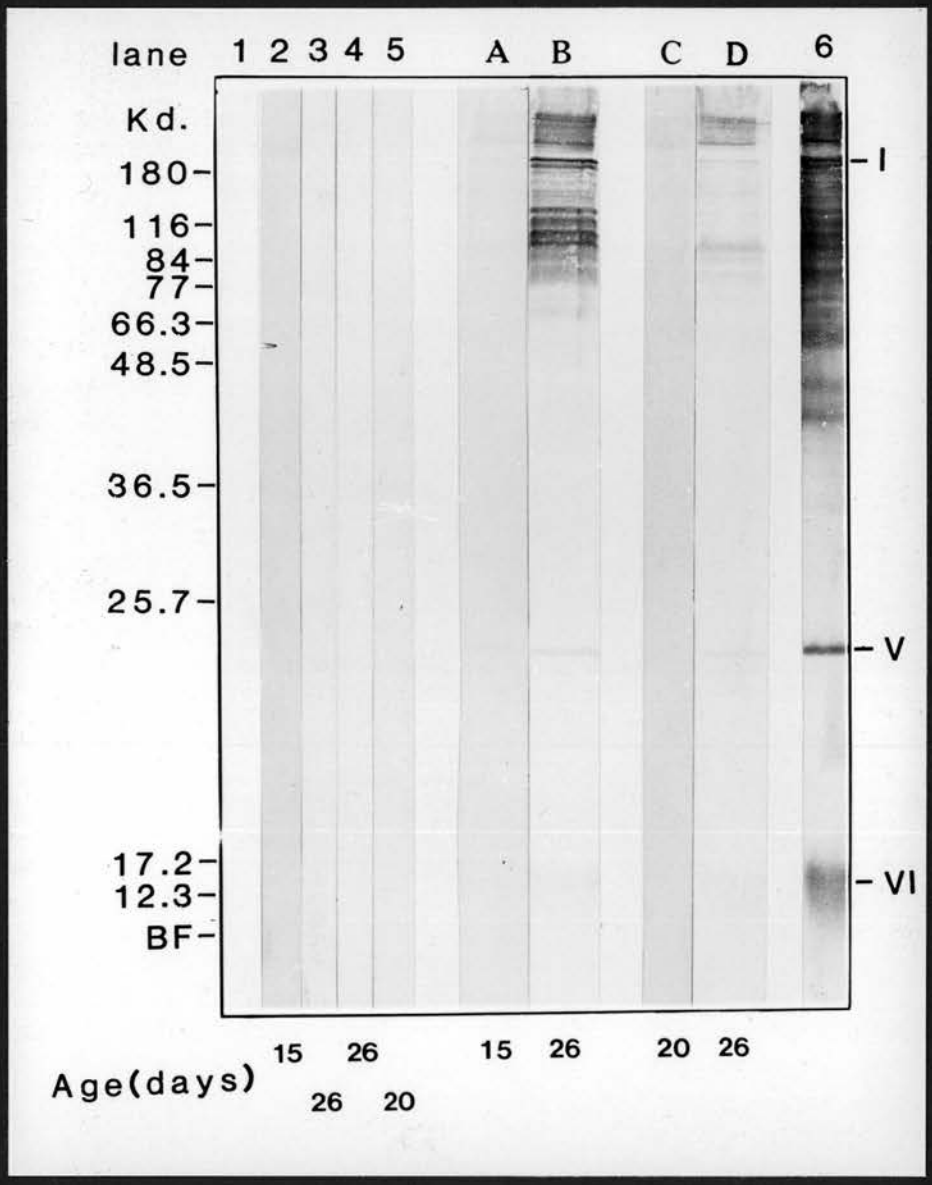

Mean IFA titre (log_n)
7
6
5
4
3
2
1
0
A
B
C
D
E
F
4
9
15
17
20
23
26
31
37
Age (days)
Mean IFA titre (log_n)
5
4
3
2
1
0
G
4
9
15
26
37
Age (days)

Figure 5.10. The specificity of antibodies in rat sera for *Cryptosporidium* oocyst antigens after primary infection at 4 or 15 days of age.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using rabbit antisera to rat immunoglobulin (Table 2.3). Antigens: I (180 Kd., double band); V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

Lane 1, conjugate control; lanes 2-5, sera from age-matched uninfected control rats; lane 6, hyperimmune rat sera (Table 2.2c); lanes A, B, C and D, pooled sera from infected rats at the times indicated by the corresponding letters in Figure 5.9. All sera were used at a 1:5 dilution.


lane 1 2 3 4 5 A B C D 6
Kd.
180
116
84
77
66.3
48.5
36.5
25.7
17.2
12.3
BF
I
V
VI
Age(days) 15 26 26 20 15 26 20 26

days of age after having been infected twice, first at 4 days and again at 15 days of age (lane B, Figure 5.10).

Immunoblot analysis of pooled convalescent serum specimens from rats at 37 days of age is shown in lanes E, F and G (Figure 5.11). Despite different infection histories (Figure 5.9), the number of antigens recognized and their molecular weight range were similar for each group of rats. Reference point antigens I, V and VI were clearly defined on each immunoblot.

Immunoblot analysis of the same pool of hyperimmune rat serum (Table 2.2c) is shown in both Figure 5.10 (lane 6) and Figure 5.11 (lane 4). Neither hyperimmune nor convalescent rat sera in this study clearly recognized any antigens between V and VI.

Section 3. SDS-PAGE and immunoblot analysis of *Cryptosporidium* merozoite antigens

Hyperimmune rabbit and sheep sera

The merozoite antigens (cervine isolate), reactive with hyperimmune rabbit and lamb sera raised against oocyst antigen are shown in Figure 5.12. Hyperimmune rabbit sera, raised against cervine or bovine oocysts (Table 2.2a), detected extensive and indistinguishable antigen profiles. Antigens with molecular weights corresponding to V and VI described earlier with oocyst antigen were distinguished using merozoite antigen. Although other discrete antigen bands were detected, they appeared diffuse compared to those produced with oocyst antigen in Figure 5.2. A similarly diffuse reaction was produced by hyperimmune lamb serum where antigens corresponding to V and VI were clearly detected. As with immunoblots of oocyst antigen described previously, the hyperimmune rabbit and lamb sera used here did not clearly detect any antigens between V and VI.

Figure 5.11. The specificity of antibodies in rat sera for *Cryptosporidium* oocyst antigens after single or multiple infections.

Antigen derived from 10^7 oocysts was applied to each lane. All lanes were developed using rabbit antisera to rat immunoglobulin (Table 2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2kD.). BF, buffer front.

Lane 1, conjugate control; lanes 2 and 3, sera from age-matched uninfected control rats; lane 4, hyperimmune rat sera (Table 2.3); lanes E, F and G, pooled sera from infected rats at the times indicated by the corresponding letters in Figure 5.9. All sera were used at a 1:5 dilution.

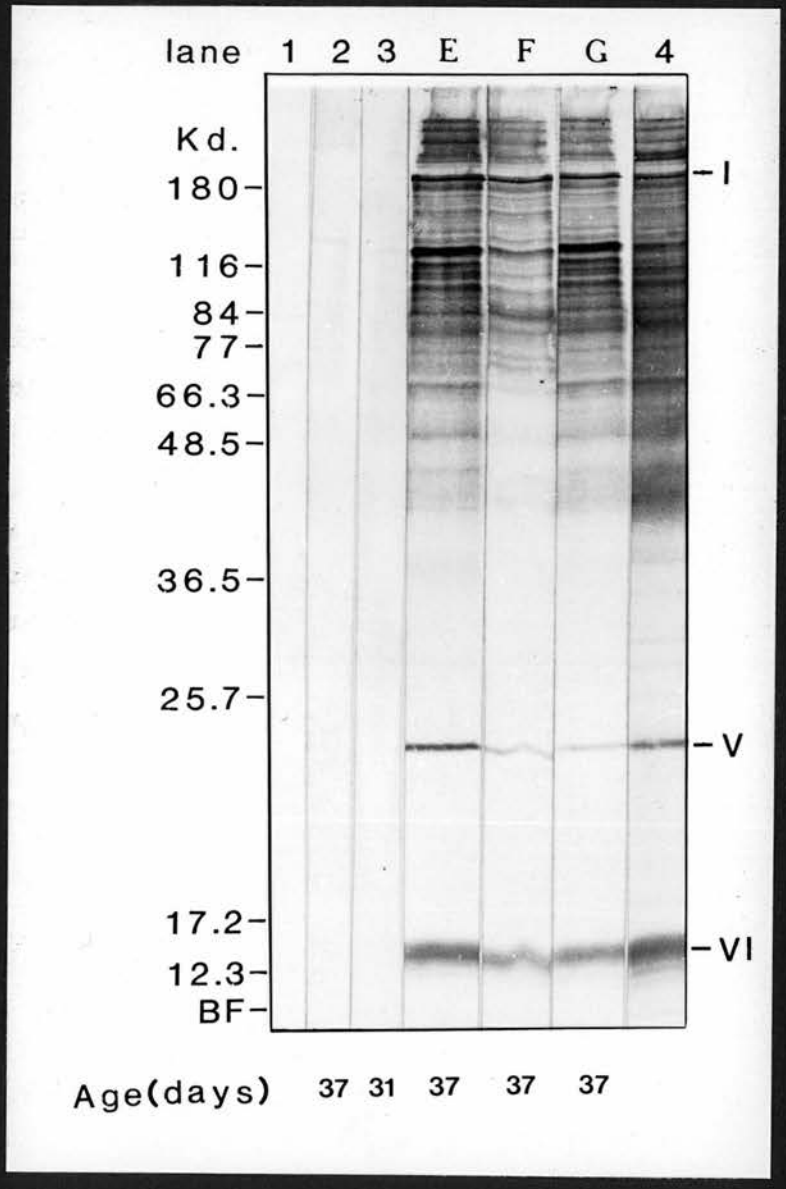

lane 1 2 3 E F G 4
Kd.
180
116
84
77
66.3
48.5
36.5
25.7
17.2
12.3
BF
I
V
VI
Age(days) 37 31 37 37 37

Figure 5.12. Immunoblots of *Cryptosporidium* merozoite antigen developed with hyperimmune rabbit and lamb sera raised against oocyst antigen.

Antigen containing $2.5x10^8$ merozoites (total protein content unknown) was denatured in 360µl of sample buffer and loaded into a 55mm wide sample well. After electrophoresis and blotting to nitrocellulose, contiguous strips were cut and reacted with the following: lanes 1 and 2, conjugate controls for pig anti-sheep $F(ab)_2$ and pig anti-rabbit immunoglobulin (Table 2.3) respectively; lanes 3 and 4, 1:10 dilutions of uninfected control lamb serum at 5 days of age and normal rabbit serum respectively; lanes 5-8, hyperimmune rabbit sera (Table 2.2a) raised against *Cryptosporidium* oocyst antigen (lanes 5 and 6, cervine isolate; lanes 7 and 8, bovine isolate); lane 9, 1:20 dilution of hyperimmune lamb serum (Table 2.2b) raised against oocyst antigen. Reciprocal serum dilutions are shown at the bottom of the figure.

Antigens: V (23Kd.); VI (12.3-17.2Kd.). BF, buffer front.

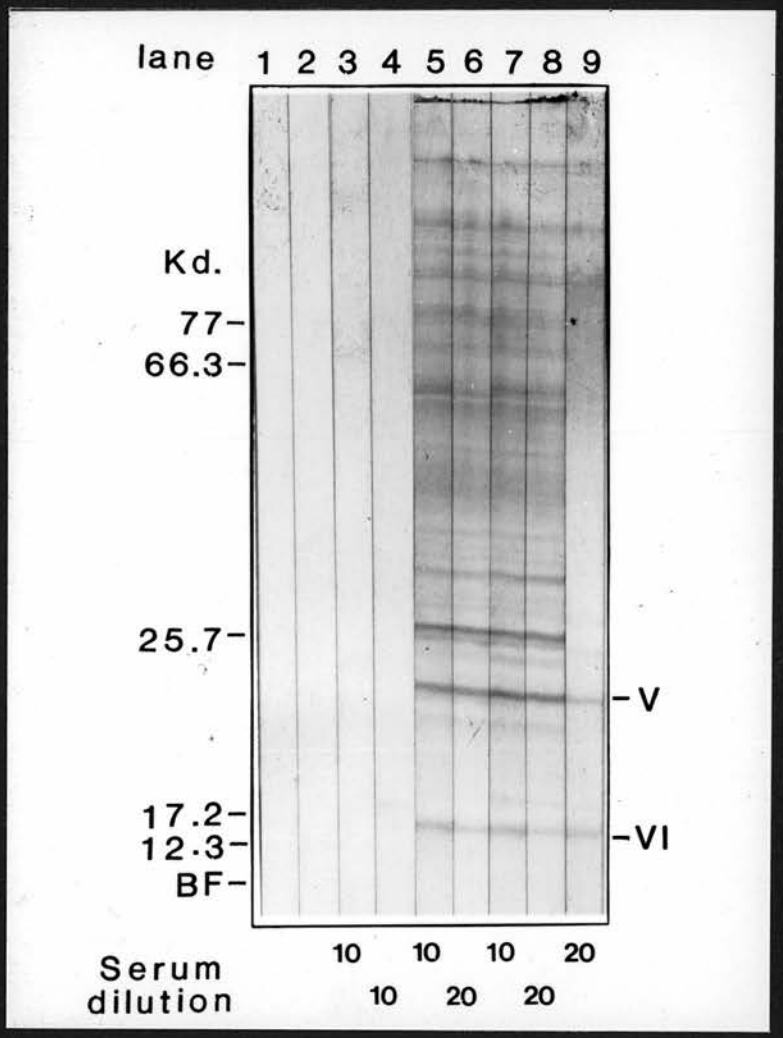

lane 1 2 3 4 5 6 7 8 9
Kd.
77-
66.3-
25.7-
17.2-
12.3-
BF-
-V
-VI
Serum dilution
10 10 10 20
10 20 20

Figure 5.13. Immunoblots of *Cryptosporidium* merozoite and oocyst antigens developed with faecal extracts from lambs with or without cryptosporidiosis.

All lanes were developed using pig antisera specific for sheep IgA (Table2.3). Antigens: I (180Kd., double band); V (23Kd.); VI (12.3-17.2Kd.).

(a) Control merozoite antigen (Con) (Section 2.9.7), containing sediments collected from intestinal washings of normal mice, was applied to lanes 1-4.

Merozoite antigen (Mer) (Section 2.9.7), containing merozoite rich sediment from intestinal washings of *Cryptosporidium* infected mice, was applied to lanes 5-9. Antigen from $2x10^7$ merozoites was loaded per lane.

Lanes 1 and 5, conjugate control; lanes 2 and 6, faecal extract from an uninfected control lamb at 4 weeks of age; lanes 3 and 7 and lanes 4, 8 and 9, pre- and 16 day post-infection faecal extracts respectively.

Oocyst antigen (Oo), derived from 10^7 oocysts, was applied to lane 10 and reacted with 16 day post-infection faecal extract as above.

(b) The antigen loadings in lanes 1, 2 and 3 were equivalent to those derived from 2x, 4x and $8x10^7$ merozoites respectively. For comparison lane 4 was loaded with oocyst antigen equivalent to that derived from 10^7 oocysts.

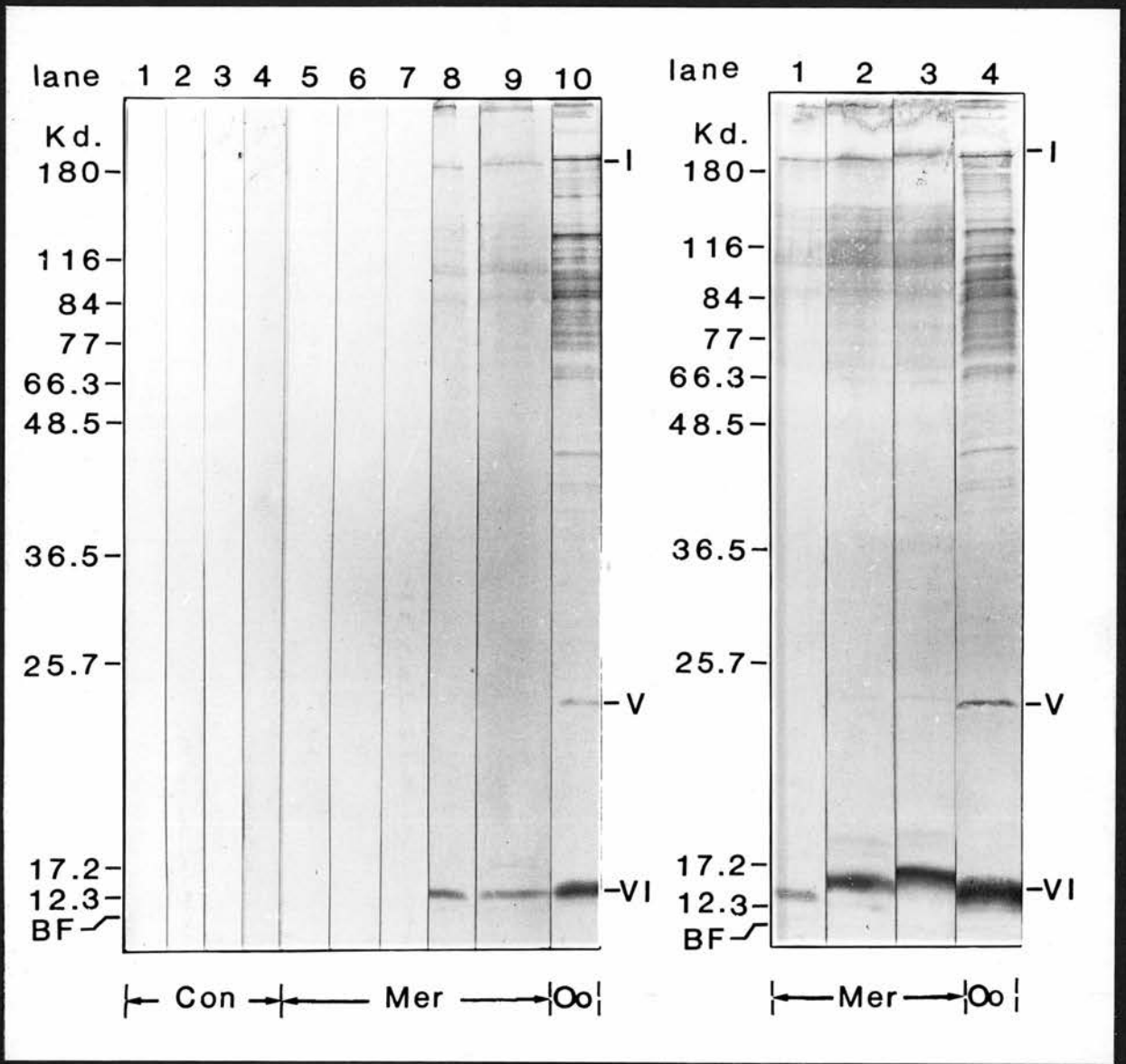


Figure 5.13a

Figure 5.13b

Faecal extracts from experimental lambs

Of the merozoite antigens recognized by IgA in faecal extract, many had apparent molecular weights greater than 66Kd. However, the reaction was diffuse and few were clearly defined (Figure 5.13a). Merozoite antigens with approximate molecular weights of 180Kd. (double band) and between 12.3 and 17.2Kd. corresponded with antigens I and VI described previously in immunoblots of oocyst antigen. Sediment from the intestinal washings of normal mice was used as a control for the merozoite rich preparation collected from washings of an equal number of infected mice. Control intestinal sediment did not react with either pre- or post-infection faecal extracts from lambs (Figure 5.13a).

As the loading of merozoite antigen per lane increased, the reactivity with IgA became more intense but the molecular weight range and definition of recognized antigens did not alter. The antigen loadings in lanes 1, 2, and 3 of Figure 5.13b were equivalent to that derived from $2x10^7$, $4x10^7$ and $8x10^7$ merozoites respectively. For comparison, lane 4 was loaded with oocyst antigen equivalent to that derived from 10^7 excysted oocysts (approximately $4x10^7$ sporozoites and their oocyst walls). An antigen with molecular weight corresponding to V(23Kd.) was not recognized by IgA in Figure 5.13b.

Discussion

This is the first report of the relationship between the shedding of *Cryptosporidium* oocysts, secretion of specific IgA and its use in immunoblotting procedures to investigate recognition of antigens which may be important to immunity.

By virtue of their consistent and conspicuous appearance six antigens (I-VI) were used, in addition to molecular weight markers, as reference points in descriptions of immunoblots in this study.

The numerous common bands between immunoblots of oocyst antigen performed with hyperimmune rabbit sera indicated a high degree of antigenic similarity between the bovine, cervine and equine isolates of *Cryptosporidium* used in Experiment 3.1 (Chapter 3). On visual assessment there was only one band which did not occur in all antigen profiles. However, antigens unique to either the bovine or equine isolates would not have been detected since all immunoblots were performed using antigen prepared from the cervine isolate. In contrast to these results, Lazo *et al* (1986) detected only 5 bands on immunoblots of a bovine isolate of *Cryptosporidium* oocysts after probing with hyperimmune rabbit serum. This difference could be explained by deterioration of their antigen which was prepared by prolonged mechanical disruption of oocysts in the absence of proteolytic inhibitors, and their immunization protocol which only used soluble antigen.

The present study showed that most of the oocyst antigens recognized by convalescent lamb sera had molecular weights greater than 45Kd. Among these a double band (I) and three single bands (II, III and IV) were most prominent and had estimated molecular weights of 180(I), 93(II), 67(III) and 47(IV)Kd. These prominent antigens were separated by a ladder-like series of minor bands.

Several *Cryptosporidium* oocyst antigens in the 66-180Kd. molecular weight range have been shown to have complex carbohydrate moieties (Luft *et al*, 1987). The efficiency of different electroblotting procedures in transferring these suspected glycoproteins may explain varying accounts of antigen recognition in this high molecular weight range. Lazo *et al* (1986) reported inefficient transfer of proteins located near the origin of the gel but recognition of 9 antigens above 60Kd. by convalescent serum from a calf infected with *Cryptosporidium*. Ungar and Nash (1986) reported recognition of only 3 antigens in this region by convalescent human sera; all had apparent molecular weights greater than 125Kd.

Numerous antigens were consistently recognized between 2 and 6 weeks post infection by serum antibodies from conventional lambs. The intensity of the reaction did not decline during this period, supporting the serological data in Chapter 4 where IFA titres did not decrease over the same period, even though oocysts were no longer detected in faeces beyond 16 days after infection. Together, these results suggested that persistent antigenic stimulation occurred through an undetected reservoir of infection.

The specificity of IgM in lamb sera included a wider range of oocyst antigens at 10 days after infection compared to that of IgG at 15 days. In contrast, immunoblots performed with faecal extracts at 15 days after infection showed that IgA reacted with a very similar range of antigens to that detected by serum IgM at 10 days. These differences in isotype specificity may reflect the relative abundance of various immunocyte precursors in the intestinal lamina propria and the range of antigens presented to them.

Oocyst antigens with estimated molecular weights of 23Kd. (single band, V) and between 12.3 and 17.2Kd (broad band, VI) were consistently recognized by serum and coproantibody from 10 days after infection. Interestingly, no clearly staining bands were detected between antigens V and VI despite numerous polypeptides staining with Coomassie blue in this molecular weight region. Recognition of an antigen with molecular weight corresponding to VI has not been reported previously. The 23Kd. antigen (V) was probably similar to that reported in studies of cryptosporidiosis in humans (Ungar and Nash, 1986). However, sera from mice experimentally infected with *Cryptosporidium* oocysts, either orally or by intraperitoneal injection, did not recognize a 23Kd. antigen (Luft *et al*, 1987).

Pooled sera from Lister rats infected orally with *Cryptosporidium* oocysts at 15 days of age showed more intense reactivity on immunoblot analysis compared to rats infected at 4 days. This supported serological results in Chapter 4 where sera from the older rats were found to have higher IFA titres. In contrast to findings with *Cryptosporidium* infected mice (Luft *et al*, 1987) both convalescent and hyperimmune sera from the rats in the present study clearly recognized a 23Kd. antigen (V). The molecular weight range of the antigens recognized by this rat sera closely matched that of convalescent lamb sera. Due to technical difficulties in collecting faeces and intestinal secretions from infant rats, the specificity of their coproantibody was not examined.

Immunoblots performed in the present study provided a detailed analysis of *Cryptosporidium* oocyst antigens recognized by hyperimmune and convalescent sera from rabbits, lambs and rats. Oocysts are the most readily available source of *Cryptosporidium* antigen but little is known about how similar they are, in terms of antigen composition, to other stages of the parasite encountered during the infection. This comparison may be especially relevant to merozoites which are encountered by the host in large numbers during the parasite's asexual phase. Studies with *Eimeria* spp. in chickens (Long and Millard, 1968; Rose and Hesketh, 1976; McDonald, Wisher, Rose and Jeffers, 1988) suggest that the asexual schizogenic stages are more immunogenic than the sporozoite and sexual stages, probably because of differences in antigen composition. Similarly, in an investigation of *E. bovis*, Reduker and Speer (1986) found differences in the antigenic composition of the sporozoite and first generation merozoite.

In preparing merozoite antigen for SDS-PAGE (Chapter 2.9.7.), no attempts were made to separate different generations of the asexual phase. Collection of merozoites from the mouse intestine reduced the likelihood of interference by extraneous

non-merozoite proteins on immunoblot analysis. If merozoites had been collected from lamb or calf intestine, then host immunoglobulins may have been included in the antigen preparation and subsequently detected as false merozoite bands by detecting antisera. Merozoites, collected for use as SDS-PAGE antigen, were free of contamination from other endogenous stages on phase contrast microscopic examination.

When hyperimmune rabbit sera, raised against oocyst antigens, were reacted with blots of merozoite antigens, the reaction covered a similar molecular weight range to that seen with oocyst antigen and many bands appeared to be shared. Serological evidence in Chapter 4 showed that IgA was a major antibody appearing in the intestinal secretions of lambs during cryptosporidiosis. Most of the merozoite antigens recognized by IgA appeared in a molecular weight range from 66-180Kd., as did most of the oocyst antigens recognized by this antibody. Immunoblots of merozoite antigen performed with faecal extracts were distinctive, in that IgA did not detect a 23Kd. antigen (V). This antigen was shown to be a consistent feature on immunoblots of oocyst antigen in the present study. Furthermore, immunoblots of merozoite antigen performed with hyperimmune rabbit sera raised against oocyst antigen indicated that an antigen with molecular weight corresponding to V was present in merozoites. This apparent conflict may be due to merozoites containing less of the antigen compared to sporozoites but in sufficient amount to enable detection by hyperimmune rabbit sera. In addition, several oocyst antigens recognized by IgA between 45 and 66Kd. were not detected by this antibody on merozoite blots.

In the present study, serum immunoblots from individual lambs at the same stage in the infection produced uniform antigen profiles whereas those performed with coproantibody gave variable profiles. Antibodies appearing in serum are part of a relatively closed system compared to the dynamics of dilution

and degradation occurring in the bowel. For this reason, immunoblots performed with coproantibody from several animals should probably be viewed as a composite when describing antigens recognized at any one time in the infection. Ascribing importance to the presence or absence of antigens in the reactions of individual faecal extracts may be misleading.

CHAPTER 6. The effects of serum and intestinal mucus on *Cryptosporidium* sporozoites

Introduction

Antibody with specificity for endogenous stages which are either attached to enterocytes or free in surface mucus may influence the course of crytposporidiosis. The interaction between intestinal mucus and motile stages of *Cryptosporidium* has not been examined, but experiments with mice and lambs in Chapter 3 showed that the amount of mucus increased during the infection. *In vivo* and *in vitro* experiments described in this chapter investigate the effects on sporozoites of serum and mucus from lambs. The aim was to examine specific and non-specific mechanisms which may be important to the host in overcoming *Cryptosporidium* infection.

Section 1. *In vitro* assessment of the effects of serum and mucus on *Cryptosporidium* sporozoites.

Materials and methods

The effects, on sporozoites, of incubation at 37^{0}C in dilutions of mucus extracts or sera from immune and non-immune lambs were assessed by measuring sporozoite/shell ratios and percentage agglutination. Sporozoite/shell ratio was used as a measure of sporozoite lysis. Values between 3 and 4 were considered normal. Loss of sporozoites from an excystation mixture was reflected in a decreased sporozoite/shell ratio. The methods used are described in detail in Chapter 2.11.1. Hyperimmune lamb serum (Table 2.2b) and sera from uninfected conventional (28 days of age) and gnotobiotic (5 days of age) lambs were tested either untreated or after inactivation (56^{0}C for 30 minutes). The colonic mucus extracts tested, from a gnotobiotic lamb 16 days after infection and an age-matched

uninfected lamb, were the same as those described in Chapter 4, Section 1. Fresh excystation fluid (Chapter 2.4) was used as control medium and the diluent for mucus extracts and sera.

Results

Section 1.1. Untreated sera

Changes in the sporozoite/shell ratio of excysted oocysts during 30 minutes incubation in sera from hyperimmunized or normal lambs are shown in Figure 6.1. The ratio decreased from values of between 3 and 4 to less than 1 after 10 minutes incubation in sera from the uninfected lambs. Ratios did not decrease as rapidly with hyperimmune lamb serum and values did not fall below 1 until 30 minutes incubation. The initial excystation percentage in all tests was at least 90% and this did not change over the incubation period, regardless of which serum was used.

After 10 minutes incubation in sera the morphology of sporozoites had changed from the normal sickle shape to a more rounded and stumpy appearance. Their cytoplasm appeared granular and vacuolated and numerous lysed, fragmented sporozoites were observed. After 30 minutes incubation in sera from either uninfected or hyperimmunized lambs, few sporozoites remained intact. Intact sporozoites showed only the morphological changes described above. Sporozoites incubated in excystation fluid alone appeared morphologically normal throughout the observation period.

At serum dilutions greater than 1:10 the proportion of sporozoites surviving was progressively increased (Figure 6.2a). At a dilution of 1:100 the sporozoite/shell ratios had values of at least 3 for hyperimmune serum and serum from an infected 28 day old conventional lamb. However, at this dilution the sporozoite/shell ratios after exposure to sera from three 5-day old gnotobiotic lambs were still less than 1 with the majority

Figure 6.1 The kinetics of *Cryptosporidium* sporozoite lysis, assessed on the basis of sporozoite/shell ratio, using hyperimmune and normal lamb sera. Excystation fluid (*---*) (Chapter 2.4) was used as control medium and to make 1:10 dilutions of hyperimmune (□) (Table 2.2b), uninfected conventional (△) (28 days of age) and uninfected gnotobiotic lamb sera (○) (5 days of age). The solid symbols (■, ▲ and ●) represent corresponding values after heat inactivation of the above sera. Values at 10, 20 and 30 minutes incubation represent the means (±SEM) from 3 separate tests. The value at 0 minutes incubation is the mean (±SEM) of all the above tests at this time.

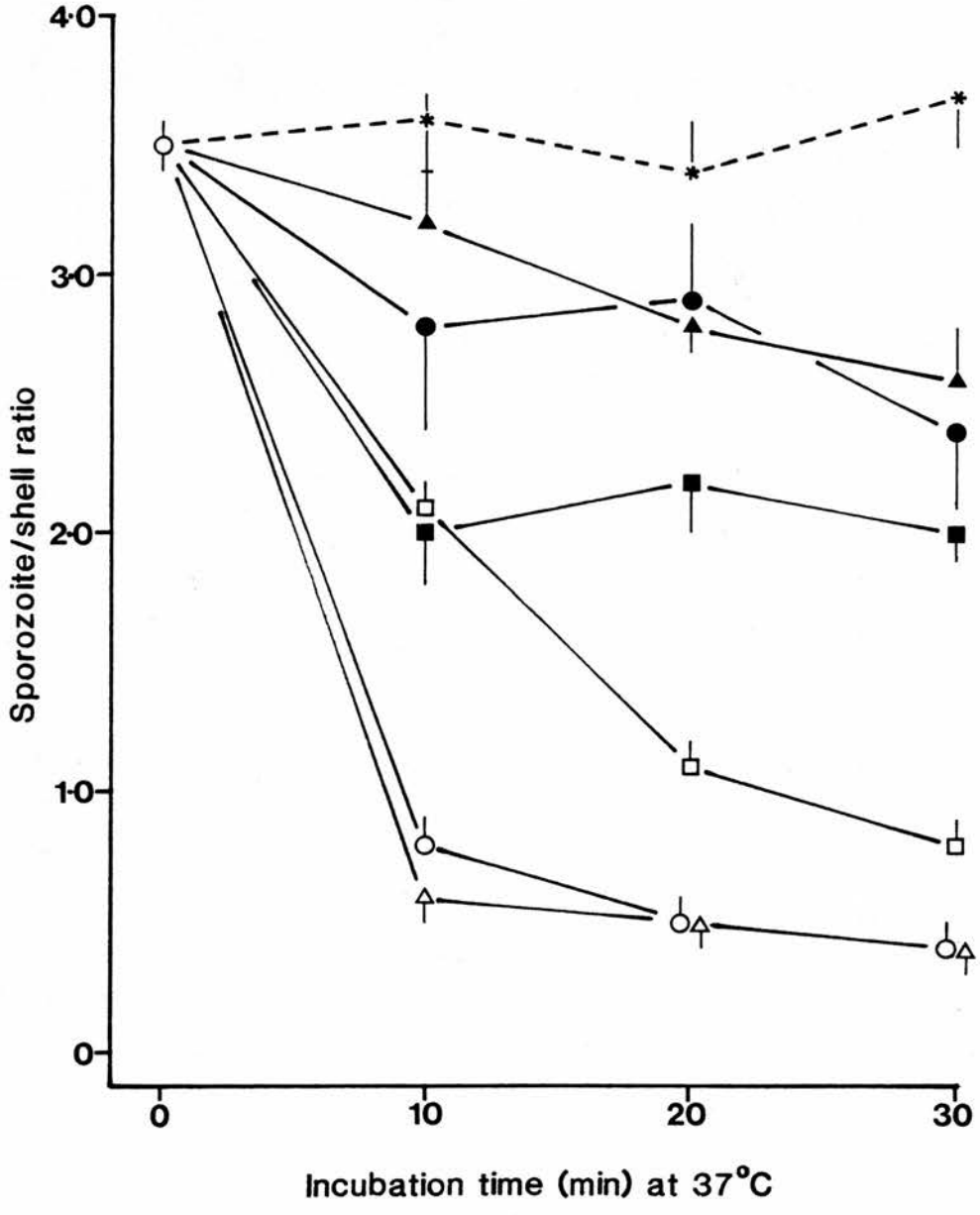

4·0
3·0
2·0
1·0
0
Sporozoite/shell ratio
0
10
20
30
Incubation time (min) at 37°C

Figure 6.2. Titration of the sporozoite lytic capacity of heat-inactivated and untreated lamb sera. The sporozoite/shell ratio was measured at 0 minutes (*---*), in excystation fluid as control medium, and the mean(±SEM) is given for the number of sera tested at each dilution.

6.2a. The sporozoite/shell ratios measured after 30 minutes are single values for dilutions of hyperimmune (□---□), uninfected conventional (△---△, 28 days of age) and uninfected gnotobiotic lamb sera (○---○, 5 days of age) in excystation fluid.

6.2b. The solid symbols (■, ▲, ●) represent corresponding values after heat inactivation of the above sera.

Figure 6.2a

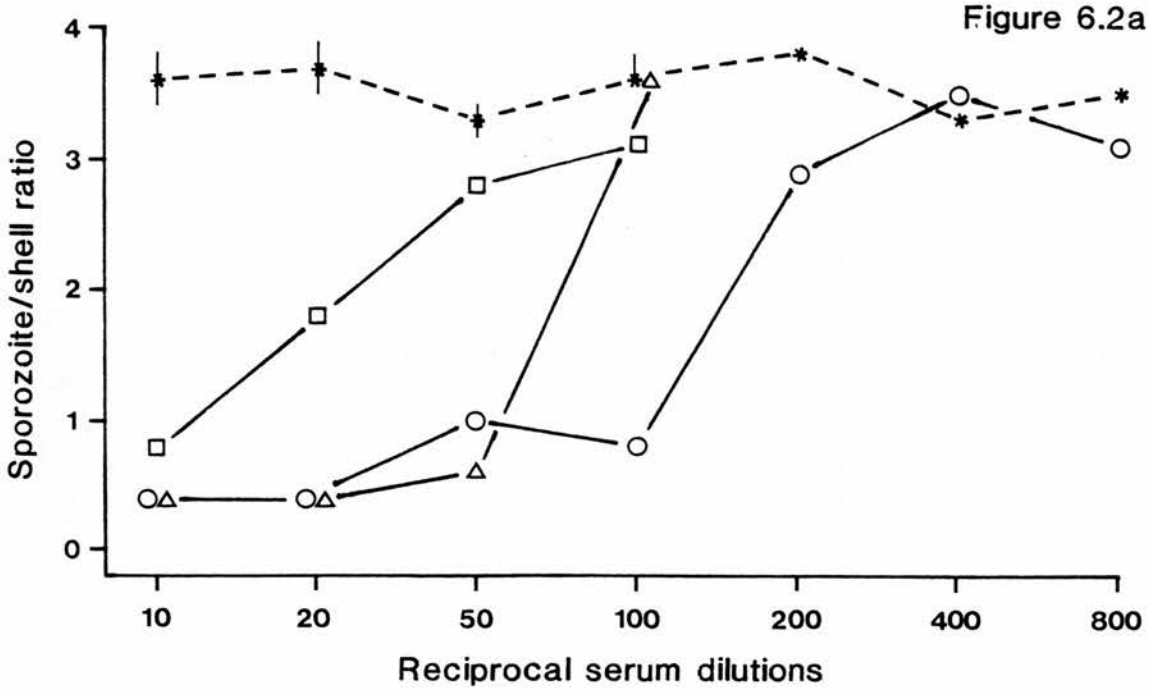
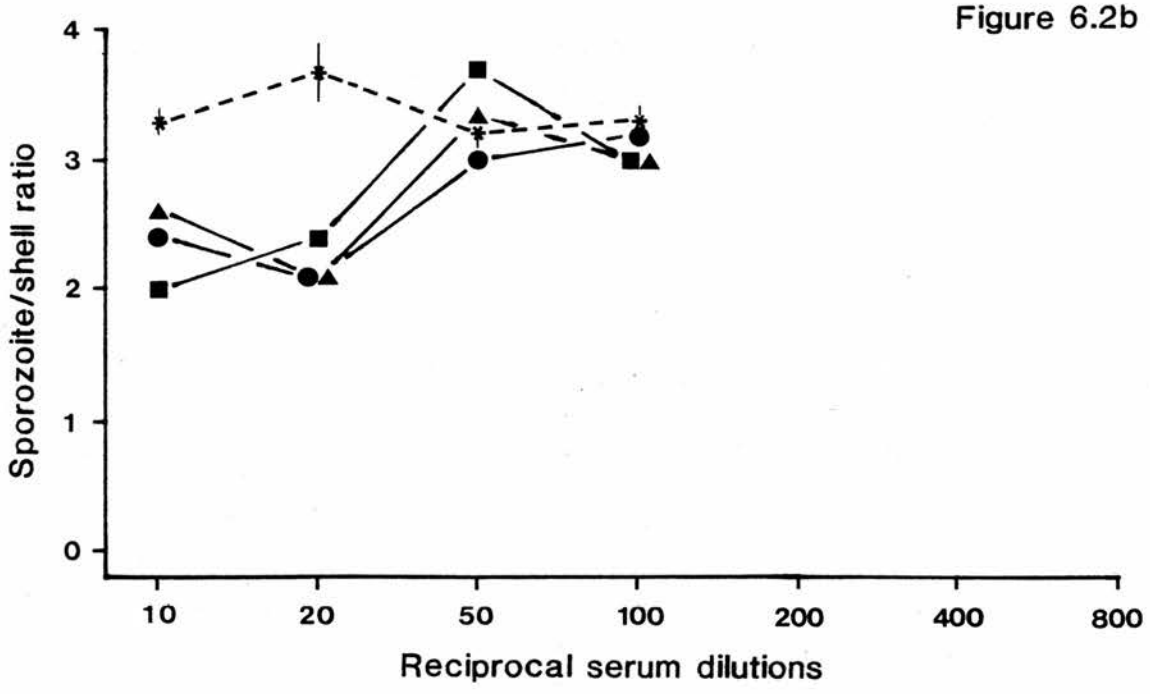


Figure 6.2b



of sporozoites fragmented. Serum from one gnotobiotic lamb was titrated further and at dilutions of 1:400 and greater the sporozoite/shell ratios were at least 3.

Section 1.2. Heat-inactivated sera

Compared to untreated sera a greater proportion of sporozoites survived exposure to heat-inactivated sera from both uninfected and hyperimmunized lambs (Figure 6.1). Sporozoite/shell ratios after exposure to heat-inactivated sera increased to at least 3 at dilutions of 1:50 and greater (Figure 6.2b).

Agglutination of sporozoites was assessed in heat-inactivated hyperimmune and uninfected gnotobiotic sera (Table 6.1). The group mean percentage agglutination, mainly in the form of sporozoite pairs, was greatest in hyperimmune serum.

Table 6.1 Agglutination of sporozoites in heat-inactivated hyperimmune or uninfected gnotobiotic lamb sera.

	Number of sporozoites (±SEM) out of 250 found as:			
	(A) Singles	(B) Pairs	(C) Three or more	%agglut-ination[2]
Excystation fluid[1]	248+1.2	2+1.2	0+0	0.8+0.5
Gnotobiotic serum	231+7.6	17+5.7	2+2	7.5+3.1
Hyperimmune serum	162+4.2	70+1.2	18+3.5	35.2+1.7

1. Excystation fluid (Chapter 2.4) was used as control medium and to make 1:10 dilutions of hyperimmune lamb serum (Table 2.2b) and serum from a 5-day old gnotobiotic lamb.
2. Group mean percentage agglutination (±SEM) was calculated from the fraction : (B+C)/250.

Section 1.3. Lamb colonic mucus fractions

No morphological changes were observed in sporozoites incubated in whole colonic mucus extracts prepared from a gnotobiotic lamb 16 days after infection and from an age-matched uninfected control. Sporozoite agglutination was a feature with both extracts but was not quantified. For further examination, extracts derived from equal weights of crude colonic mucus from these lambs were fractionated by gel filtration on a Bio-Gel A-1.5m column, as described previously in Chapter 4, Section 1. In addition to the distribution of glycoprotein and specific antibody, as detected by analysis for hexose and IFA respectively, Figure 6.3 shows the assessment of sporozoite agglutination by the mucus fractions.

Agglutinated sporozoites were detected in all 7 fractions from the infected lamb but only in fractions 1-3 from the uninfected control lamb(Figure 6.4). Agglutination in control mucus was associated with those fractions having hexose concentrations greater than 1000μg/ml. The protein concentrations in mucus fractions 1-7 from the control lamb were 1.3, 1.6, 1.3, 1.5, 1.5, 2.6 and 0.7 mg/ml respectively. Protein concentrations were not measured in fractions from the infected lamb. Sporozoites in mucus fractions from either infected or control lambs showed no morphological changes after 30 minutes incubation and sporozoite/shell ratios were unchanged.

Figure 6.3. Analysis of soluble colonic mucus extracts, from a *Cryptosporidium* infected lamb and an uninfected control lamb, after fractionation on Bio-Gel A-1.5m. For details of filtration see Chapter 4, Figure 4.6. Fractions 1-7 were analysed for hexose content (6.3a) and *Cryptosporidium* specific IgA (6.3b) after each had been concentrated to 1ml. Sporozoite agglutination (6.3c) was assessed after 30 minutes incubation at 37^{0}C with mucus fractions diluted 1:2 with excystation fluid. Agglutination values are the mean of 3 separate assessments.

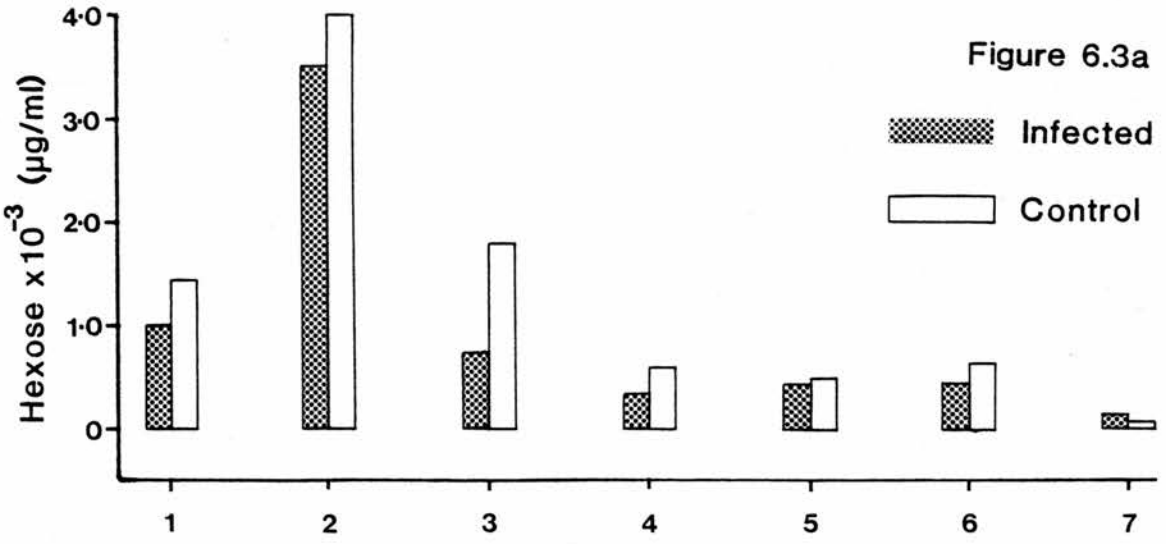


Figure 6.3a
Infected
Control
Hexose x10^{-3} (µg/ml)
4·0
3·0
2·0
1·0
0
1
2
3
4
5
6
7

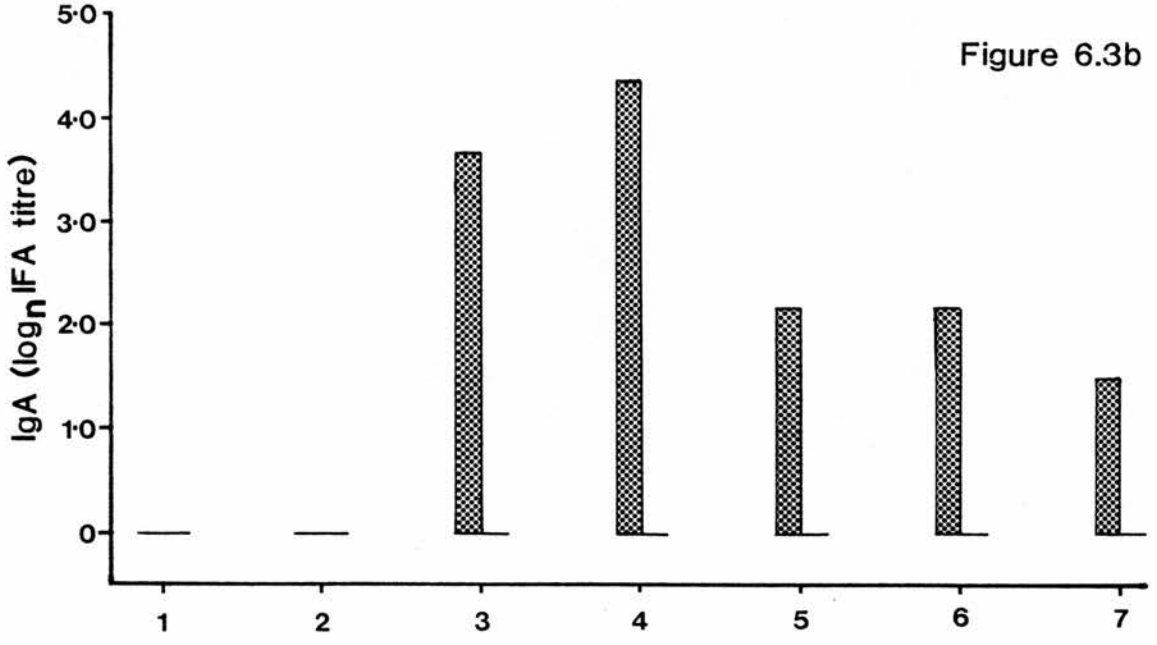


Figure 6.3b
IgA (log$_n$IFA titre)
5·0
4·0
3·0
2·0
1·0
0
1
2
3
4
5
6
7

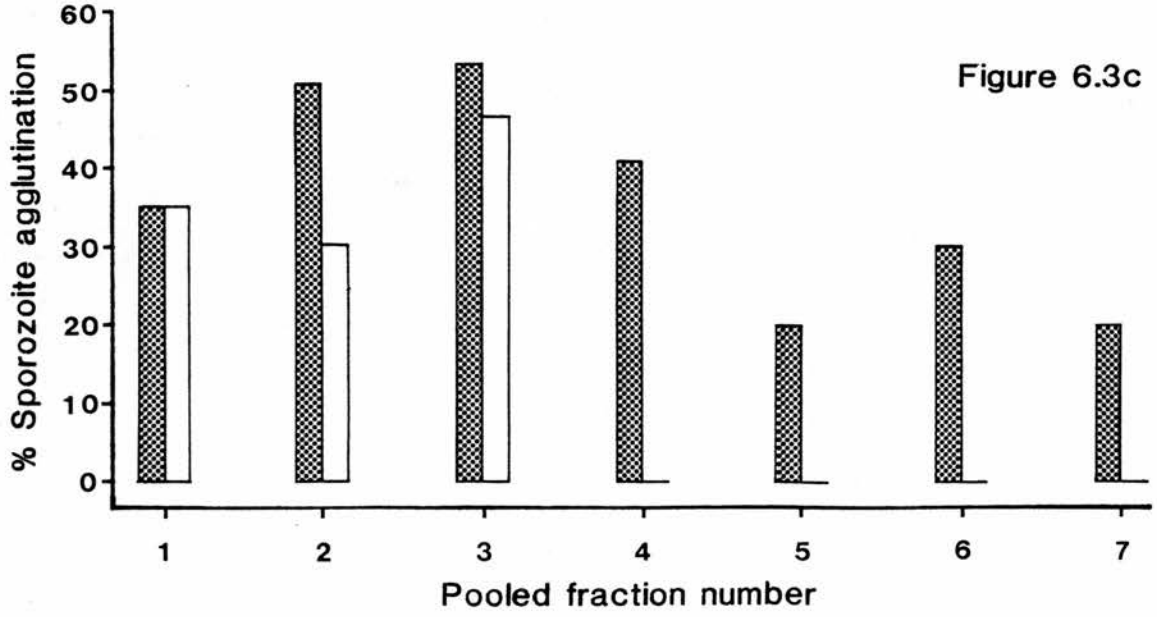


Figure 6.3c
% Sporozoite agglutination
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Pooled fraction number

Figure 6.4. *Cryptosporidium* sporozoites (small arrows) in a 1:2 dilution of Bio-Gel fraction number 3 from the infected gnotobiotic lamb described in Figure 6.3. Sporozoites exist singly, agglutinated in pairs or in groups of three or more. Oocyst walls are also shown (large arrow). Phase contrast microscopy. x1000.

Section 2. *In vivo* assessment of sporozoite infectivity.

Materials and methods

Cryptosporidium sporozoites (cervine isolate) were first incubated *in vitro* at 37^{0}C for 30 minutes in either fresh excystation fluid (Chapter 2.4) or in 1:10 dilutions of the heat-inactivated immune and non-immune sera described in Section 1 of this chapter. The infectivity of treated sporozoites was then assessed by inoculating them, per rectum, into 5-day old Lister rats using methods described in Chapter 2.11.2 and 2.11.3.

Control rats were inoculated with sporozoites incubated only in excystation fluid. In different experimental designs, controls were included either or both as whole control litters or as within litter controls. Rats were killed in half-litter groups at 3 or 5 days after inoculation. Their small and large intestines were prepared for histological examination from which the proportion of infected rats was determined. Differences in the proportion infected, between control and serum treatment groups, were tested for significance using Fisher's Exact Test (2-tailed).

Hyperimmune lamb serum (Table 2.2b) and serum from the 5-day old gnotobiotic lamb were also examined after affinity chromatography on Protein G Sepharose (Chapter 2.9.4) to remove IgG.

Results

Section 2.1. Whole sera

After inoculation of sporozoites per rectum, endogenous stages were only observed in ileum, caecum and colon.

Table 6.2 shows the proportion of rats which were infected at 3 or 5 days after inoculation for both control and serum treatment groups. All control rats were found to be infected. At the lower dose rate, of 10^4 excysted oocysts, only 2 of 14 rats became infected after sporozoites had been treated with serum. At the higher dose rate, of 10^6 excysted oocysts, the proportion of rats infected was lowest at 3 days after inoculation where sporozoites had been treated with hyperimmune serum. However, 5 days after inoculation of 10^6 excysted oocysts, there were no significant differences in the proportion of rats infected, irrespective of the source of serum.

Section 2.2. IgG-rich and -depleted sera

Preparation of serum fractions

Figure 6.5 shows the absorbance profiles for separation of IgG from hyperimmune (Table 2.2b) and uninfected control gnotobiotic lamb serum using Protein G Sepharose. Specific antibody to sporozoites was not detected by IFA with the gnotobiotic serum or its Protein G fractions. The titres of IgM and IgG on IFA of whole hyperimmune serum were 1:80 and 1:2560 respectively but specific IgA was not detected.

The distribution of total IgA, IgM and IgG in the Protein G fractions is shown in Figure 6.6. IgA was detected in the hyperimmune serum and its fall-through(FT) fraction but not in the fraction eluted from Protein G. IgA was not detected in the gnotobiotic serum or its Protein G fractions. IgG was not detected in the FT fractions of either the hyperimmune or gnotobiotic sera, but was present in large amounts in the eluted fractions. IgM was detected in the eluted fraction(ELb) of hyperimmune serum but not in the corresponding fraction(ELa) from gnotobiotic serum.

Table 6.2. *In vitro* neutralization of *Cryptosporidium* sporozoites and assessment of subsequent infectivity after inoculation, per rectum, into 5-day old Lister rats.

		Time after inoculation	
		Three days	Five days
Treatment[1]	Dose[2]	No. infected/ no. examined	No. infected/ no. examined
Control	10^6	6/6	12/12
	10^4	4/4	8/8
Hyperimmune serum	10^6	2/9 *[3]	10/12 NS
	10^4	0/9 **	0/10 ***
Conventional serum	10^6	11/11 NS	10/11 NS
	10^4	0/6 **	0/6 ***
Gnotobiotic serum	10^6	7/7 NS	8/8 NS
	10^4	0/12 **	2/14 ***

1. Fresh excystation fluid was used as control medium and to make 1:10 dilutions of hyperimmune (Table 2.2b) uninfected conventional and gnotobiotic lamb sera.

2. Each rat was inoculated with sporozoites derived from either 10^6 or 10^4 oocysts after excystation (Chapter 2.4).

3. Differences in the proportions infected between groups of rats inoculated with serum-treated sporozoites and their dose equivalent control group were tested for significance using Fisher's Exact Test (2-tailed). NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure 6.5. The use of affinity chromatography to separate IgG from serum.

Gel: Protein G Sepharose 4 Fast Flow (Pharmacia Ltd.)

Samples:

Run(a). 1ml of gnotobiotic lamb serum diluted in 2ml of PBS.

Run(b). 1ml of hyperimmune lamb serum (Table 2.2b) diluted in 2ml PBS.

Run(c). pooled fall through fraction from Run(b).

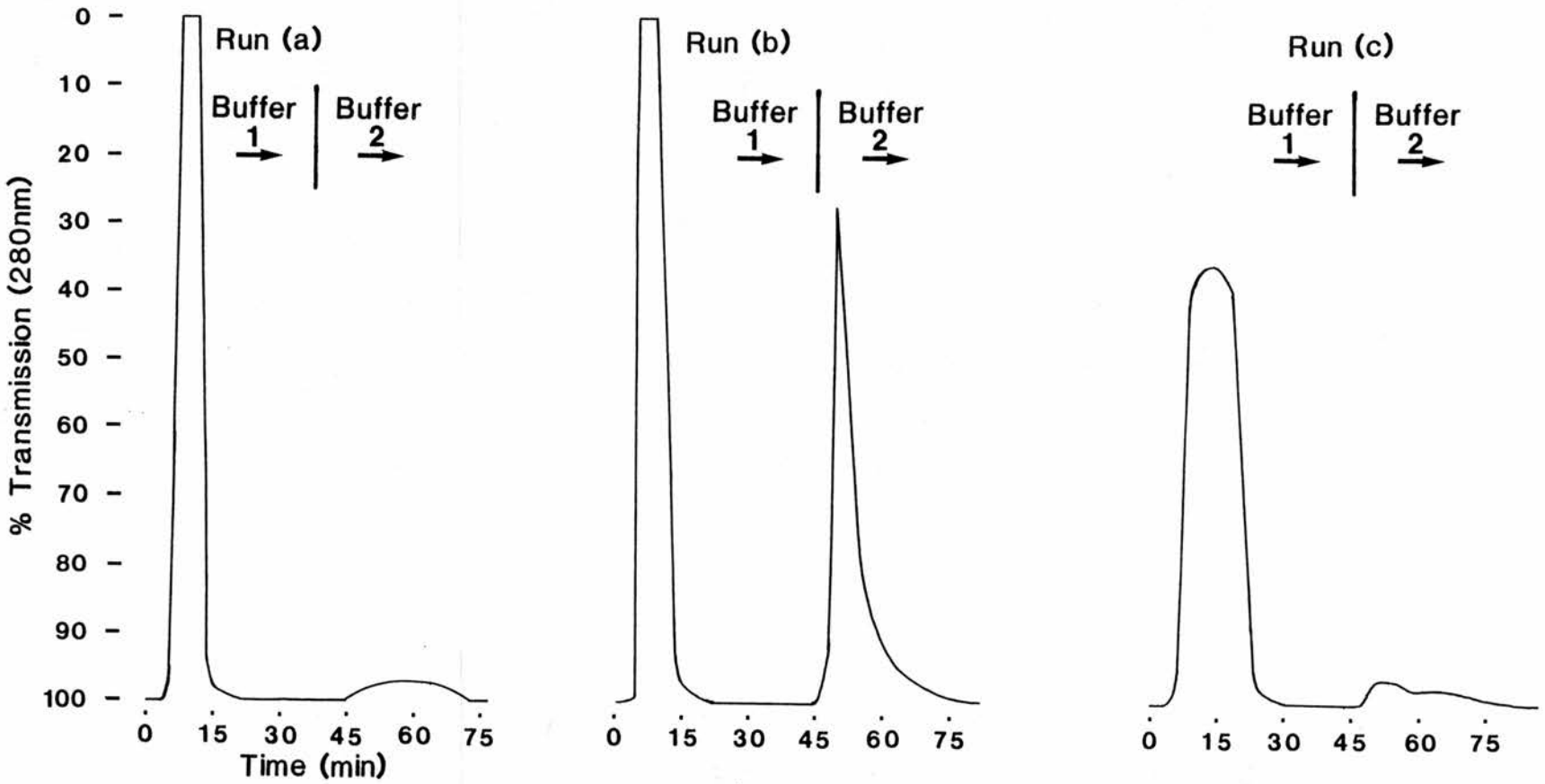
Bed dimensions: 1x7 cm. Flow rate: 0.6ml/min.

Starting buffer(1): PBS pH 7.1.

Elution buffer(2): 0.1M glycine-HCl,pH 2.7.

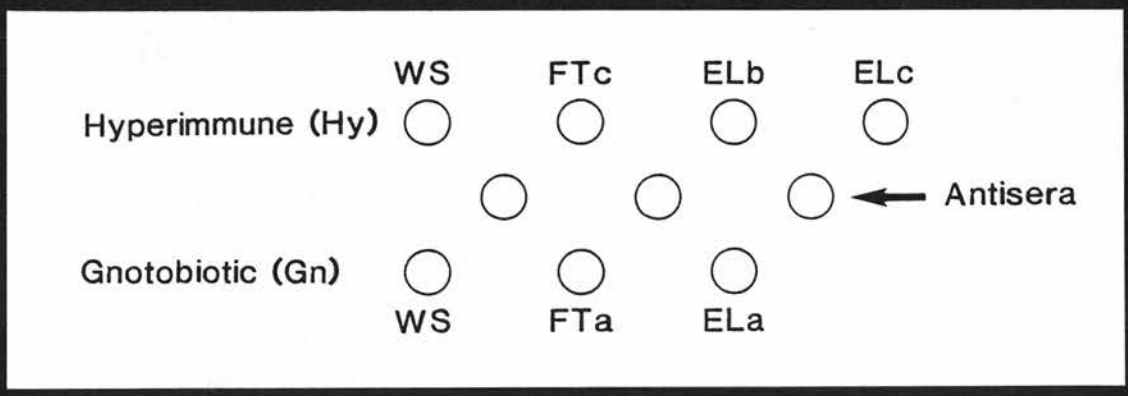
After each elution the column was re-equilibrated with starting buffer. To preserve the activity of IgG a few drops of 1M Tris-HCl ,pH 9.0, were added to eluted fractions as they came off the column so that their final pH was approximately neutral.

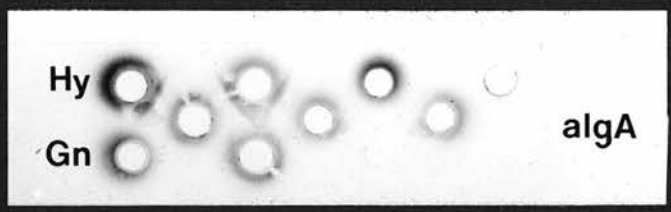
Pooled (├──┤) 5 minute fall through fractions(FT) and IgG-rich eluted fractions(EL) from each run were concentrated to 1ml by vacuum dialysis against PBS. Specific IgM, IgA and IgG isotypes to *Cryptosporidium* sporozoites were then titred by IFA. Fall through from Run(b) was not tested(nt) and fractions from gnotobiotic serum were negative(-).

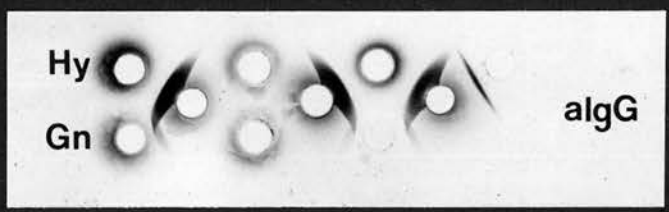


	FTa	ELa	FTb	ELb	FTc	ELc
IgM	–	–	nt	–	1:40	–
IgA	–	–	nt	–	–	–
IgG	–	–	nt	1:1280	–	1:10

Figure 6.6. Distribution of IgA, IgM and IgG in fractions of hyperimmune(Hy) and gnotobiotic(Gn) lamb sera. Antisera were denoted as aIgA, aIgG and aIgM and were used neat. Well volumes were 10µl. Fall through(FT) and IgG-rich(EL) fractions were derived from the hyperimmune and gnotobiotic lamb sera described in Figure 6.5. Whole sera(WS) were included for comparison. The schematic diagram at the top of this figure shows the well content and pattern used for each antisera.


WS
FTc
ELb
ELc
Hyperimmune (Hy)
Antisera
Gnotobiotic (Gn)
WS
FTa
ELa


Hy
Gn
αIgA


Hy
Gn
αIgG


Hy
Gn
αIgM

Immunoelectrophoresis confirmed numerous arcs of identity between the gnotobiotic serum and its FT fraction and a faint IgG arc was observed in the eluted fraction(ELa) (Figure 6.7). A similar result was obtained with hyperimmune serum and its FT fraction (Figure 6.7). Immunoelectrophoretic analysis of the eluted fraction with isotype specific antisera confirmed this complex to be largely IgG, with a small amount of IgM and no detectable IgA (Figure 6.8). This fraction also contained other unknown proteins which reacted with anti-whole serum (Figure 6.8).

The fall through fractions of the gnotobiotic(FTa) and hyperimmune(FTc) lamb sera did not contain any detectable IgG (Figure 6.6). These and the corresponding IgG-rich eluted fractions(ELa and ELb) were concentrated to the volume of the original serum samples and tested for sporozoite neutralization.

Testing of serum fractions

All control rats, both as whole litter groups and those included in serum treatment groups, were infected when examined at either 3 or 5 days after inoculation (Table 6.3).

At the lower dose rate, of 10^4 excysted oocysts, the proportion of rats infected was significantly reduced in those groups where sporozoites had been treated with IgG-depleted sera from either gnotobiotic or hyperimmune lambs. A similar reduction in infectivity was found for sporozoites treated with the IgG-rich fraction prepared from hyperimmune serum irrespective of whether rats were killed 3 or 5 days after inoculation. However, treatment of sporozoites with the IgG-rich fraction of gnotobiotic serum did not reduce the proportion of rats infected (Table 6.3).

At the higher dose rate, of 10^6 excysted oocysts, the proportion of rats which became infected was very high in each treatment

Figure 6.7. Distribution of serum proteins in gnotobiotic and hyperimmune serum after fractionation on Protein G sepharose. Troughs were filled with 100µl of pig anti-whole sheep serum (aWSS) used neat. Wells contained 10µl of fall through(FT) or eluted fractions(EL) from the hyperimmune and gnotobiotic lamb sera described in Figures 6.5 and 6.6. Whole sera(WS) were included for comparison. Arrows mark IgG(↓) arcs and those unidentified proteins in the ELb(◆) fraction. The cathode is at the top.

Figure 6.8. Distribution of sheep immunoglobulins and serum proteins in the fraction of hyperimmune lamb serum eluted from Protein G sepharose. The IgG-rich fraction(ELb) from the hyperimmune lamb serum was electrophoresed and developed with anti-whole sheep serum(aWSS) and antisera specific for sheep IgA(aIgA), sheep IgG(aIgG) and sheep IgM(aIgM). The arrowhead (▲) marks the unidentified proteins in the ELb fraction. The cathode is at the top.

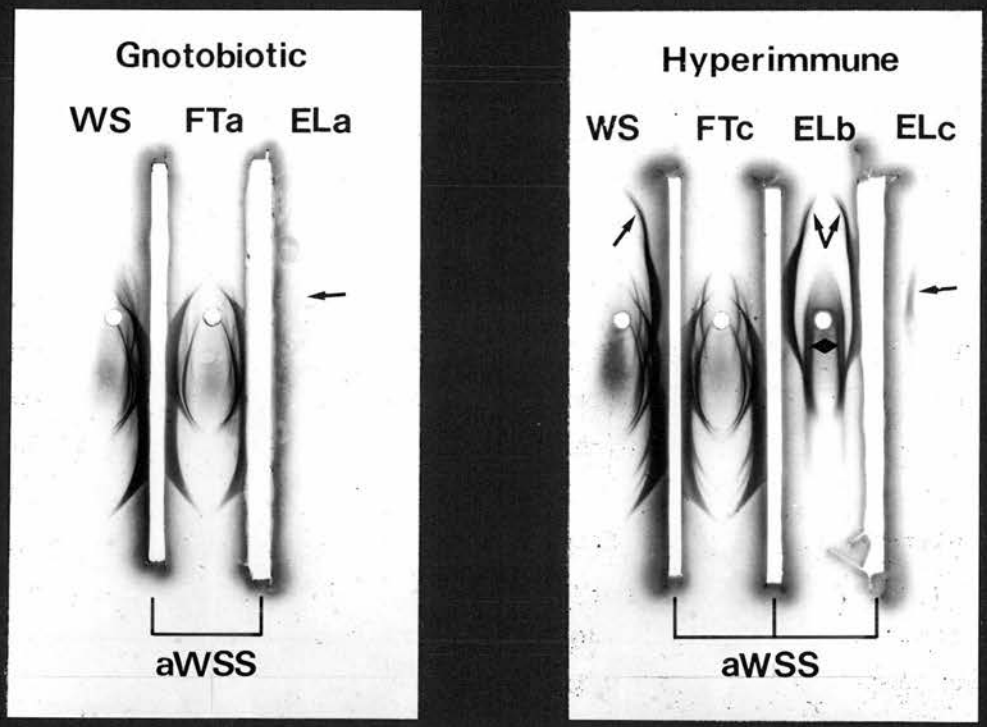


Figure 6.7

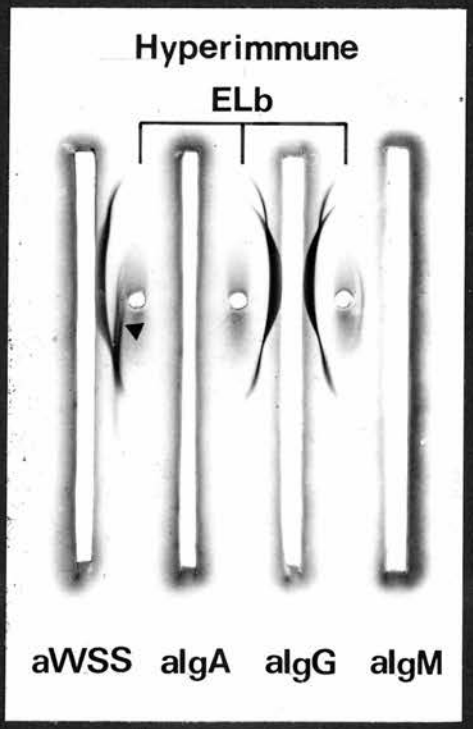


Figure 6.8

Table 6.3. Footnotes.

1. Fresh excystation fluid was used as control medium and to make 1:10 dilutions of fall through(FT) and IgG-rich eluted(EL) fractions prepared from the gnotobiotic and hyperimmune (Table 2.2b) lamb sera described in Figure 6.5.

2. Each rat was inoculated with sporozoites derived from 10^6 oocysts after excystation (Chapter 2.4) or ten-fold dilutions of this dose in excystation fluid. Control rats were included both as whole litters and within serum treatment groups.

3. Differences in the proportions infected between groups of rats inoculated with serum-treated sporozoites and their dose equivalent whole litter control groups were tested for significance using Fisher's Exact Test (2-tailed). NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 6.3. *In vitro* neutralization of *Cryptosporidium* sporozoites and assessment of subsequent infectivity after inoculation, per rectum, into 5-day old Lister rats.

Treatment[1]	Dose[2]	Time after inoculation: Three days — No. infected/ no. examined	Time after inoculation: Five days — No. infected/ no. examined
Control	10^6	9/9	9/9
	10^5	2/2	2/2
	10^4	9/9	10/10
	10^3	2/2	3/3
IgG-depleted gnotobiotic serum fraction (FTa)	10^6	4/4 NS[3]	4/4 NS
	10^4	2/14 ***	2/9 **
Control	10^6	2/2	2/2
	10^4	2/2	3/3
IgG-rich gnotobiotic serum fraction (ELa)	10^6	2/2 NS	4/4 NS
	10^4	9/9 NS	8/8 NS
Control	10^6	not done	not done
	10^4	1/1	2/2
IgG-depleted hyperimmune serum fraction (FTc)	10^6	6/7 NS	6/6 NS
	10^4	0/5 **	0/5 ***
Control	10^6	5/5	5/5
	10^4	2/2	2/2
IgG-rich hyperimmune serum fraction (ELb)	10^6	5/5 NS	5/5 NS
	10^4	1/17 ***	2/17 ***
Control	10^6	2/2	2/2
	10^4	6/6	6/6

group and not significantly different from the controls. This result was independent of whether rats were killed at 3 or 5 days after inoculation. However, at 3 days, only small numbers of *Cryptosporidium* endogenous stages were observed in those rats where sporozoites had been treated with IgG-depleted serum from either hyperimmune or gnotobiotic lambs. In contrast, endogenous stages were numerous at both 3 and 5 days in control rats and rats where sporozoites had been treated with IgG-rich fractions of hyperimmune or gnotobiotic sera.

Discussion

The diluent used for serum, mucus and sporozoites was excystation fluid (Chapter 2.4). This fluid induces rapid and high percentage *in vitro* excystation under defined pH and bile salt concentrations. It was used as a diluent because sporozoite neutralization mechanisms in the gut may have to operate under similar conditions of pH and bile salt concentration.

The effect of incubating sporozoites in sera or mucus extracts was assessed by *in vitro* and *in vivo* methods. For the *in vitro* method, the inert nature and durability of the oocyst wall enabled an excystation mixture to be monitored for changes in sporozoite numbers by means of the sporozoite/shell ratio. This ratio was similarly used in *in vitro* studies of sporulation in *Cryptosporidium* (Woodmansee, 1986).

An interesting finding was that sporozoites incubated *in vitro* with either unheated hyperimmune or non-immune sera were rapidly lysed and fragmented. At a 1:10 dilution of sera this resulted in a dramatically reduced sporozoite/shell ratio with few intact sporozoites observed. Sporozoite/shell ratios were used as a measure of sporozoite lysis. Sporozoites which appeared intact under phase contrast microscopy were included in the assessment while lysed, fragmented sporozoites were not. This reduced the difficulty of interpreting partial sporozoite reactions,

observed only as morphological changes from the normal sickle shape to a more stumpy appearance. Any continuing excystation during the observation period could have masked the full effect of serum treatment. However, this would not have seriously interfered with the results because at least 90% of oocysts had excysted at the beginning of the test.

Since *Cryptosporidium* oocysts contain 4 sporozoites, the theoretical value for the sporozoite/shell ratio would also be 4. In practice, values between 3 and 4 were found using the excystation conditions and methods of assessment described in Chapter 2.4. For experiments in this chapter, values for the ratio between 3 and 4 were considered normal.

The methods used in the present study to collect, store and excyst *Cryptosporidium* oocysts allowed sporozoite agglutination to be studied in suspensions largely free of extraneous debris. Agglutination could not be assessed in unheated immune and non-immune sera because the majority of sporozoites underwent lysis and fragmentation. Sporozoite agglutination was studied only in heat-inactivated sera used at a dilution of 1:10. Agglutination was consistently greater in the presence of hyperimmune (35±1.7%) compared to uninfected gnotobiotic (7.5±3.1%) serum and negligible in control excystation fluid (0.8±0.5%). These results contrast with the work of Riggs and Perryman (1987) where, although *Cryptosporidium* sporozoite agglutination was not specifically measured, it was described as "minimal" in hyperimmune bovine serum. However, in different parasite-host systems, merozoites of *Eimeria bovis* and *E. tenella* have been shown to be agglutinated by convalescent calf (Andersen, Lowder, Hammond and Carter, 1965) and chicken (McDermott and Stauber, 1954) sera respectively.

The present study provides the first assessment of the interaction between *Cryptosporidium* sporozoites, specific IgA and intestinal surface mucus. Sporozoite agglutination in

colonic mucus fractions from the infected lamb had specific (associated with specific antibody) and non-specific components. Thus, agglutination was detected in mucus fractions containing specific IgA, but it was also associated with fractions rich in hexose and devoid of detectable specific IgA. Similar activity, associated with the hexose-rich mucus fractions from the uninfected control lamb, supported the notion of non-specific mucus agglutination. Sporozoite agglutination in mucus fractions containing the highest concentrations of hexose suggested that mucus glycoproteins might be responsible. Fractionation of mucus, by caesium chloride equilibrium density gradient ultracentrifugation, into protein- and carbohydrate-rich fractions would be helpful in resolving this question.

For the *in vivo* method of assessing serum-treated sporozoite infectivity, intestines of recipient rats were examined 3 and 5 days after inoculation. Preliminary investigations (Table 6.2) showed that although hyperimmune serum reduced the proportion of rats detectably infected at 3 days, there was no significant difference at 5 days after inoculation. This occurred at a dose rate of 10^6 excysted oocysts per rat. Hence, it was found essential not to limit observations to 3 days after inoculation because even a thorough histological examination at this time could miss small numbers of endogenous stages. This agreed with the results of Experiment 3.2 (Chapter 3) where maximum *Cryptosporidium* endogenous stage population densities in the small and large intestines were not reached until 5 and 8 days respectively after a single inoculation.

Even though the sporozoite suspensions used in this study had excystation percentages of at least 90%, contamination with intact oocysts may have masked the full effects of serum treatment. The numerous rats in serum treatment groups found not to be infected suggested either that contaminating oocysts failed to excyst, that sporozoites excysting *in vivo* were

subsequently neutralized or that the extent of their infection was too small to be detected histologically. The sensitivity of the rat infection assay was tested at dose rates from 10^3 to 10^6 excysted oocysts per rat and over this range, infection was detected in all inoculated animals.

Treatment of *Cryptosporidium* sporozoites with heat-inactivated sera greatly reduced their subsequent infectivity in 5-day old rats; this neutralization had both specific (associated with specific antibody) and non-specific components. Specific neutralization was associated with the IgG fraction from hyperimmune serum. However, removing total IgG from hyperimmune serum did not eliminate its capacity to reduce the infectivity of sporozoites. Remaining activity could have been due to either the low titre (1:40) of specific IgM detected or the unidentified heat-stable, non-dialysable components responsible for the activity in IgG-depleted uninfected gnotobiotic lamb serum.

The reduced infectivity of serum-treated sporozoites demonstrated in the present study may apply only within the circumstances defined by these experiments. However, the reduced infectivity associated with specific IgG may have important implications for passive protection in young ruminants. It was for this reason that IgG was chosen to investigate antibody-mediated protection against cryptosporidiosis, despite the association made between increasing titres of IgA in intestinal secretions and declining oocyst output in lambs. IgG is quantitatively the most important isotype in ruminant milk, even in that from locally immunized animals where IgA levels are substantially increased (Watson and Lascelles, 1973). Furthermore, in species where enteric cryptosporidiosis causes inflammatory exudation, then the inclusion of those non-specific serum components with activity against sporozoites may influence the course of infection. These factors are discussed more fully in Chapter 7.

CHAPTER 7. General Discussion

This study had three main aims: first, to establish and investigate cryptosporidiosis in a symptomatic laboratory animal model where diarrhoea was considered the most important clinical sign; secondly, to compare and contrast this model with the infection in young ruminants in terms of parasite biology and immunology; thirdly, to determine the neutralizing ability of specific immunoglobulins using *in vitro* and *in vivo* techniques.

Rats and mice were readily available candidates for a laboratory animal model and strains of each species were experimentally infected with three isolates of *Cryptosporidium*. Using histopathological scoring methods, it was shown that the distribution and severity of infection depended more on the host species than on the isolates used in the experiment. The Lister rat was selected as a suitable laboratory animal host because infection was symptomatic with a distribution in the gut similar to that in the lamb.

Lister rats infected at 4 days of age showed a premature but transient loss of supranuclear vacuolated ileal enterocytes 8 days after infection, coinciding with severe villous atrophy. Mean villous length for infected rats was less than half that of controls in the jejunum on day 5 and in the ileum on day 8 after infection. While morphometric studies on histological sections allow assessment of the size of the proliferative compartment (length and width of the crypts), no inferences can be drawn on the proportion of the crypt-cell population that was replicating or the duration of the cell cycle. However, even if the rate of cell production and replacement remained the same after infection, the degree of villous atrophy suggested that the migration time for enterocytes along jejunal and ileal villi would be at least halved at 5 and 8 days respectively.

In the rat, the normal replacement of vacuolated ileal cells at weaning is accompanied by a dramatic reduction in villous migration time (Koldovsky *et al*, 1966; Clarke and Hardy, 1969; Cheng and Leblond, 1974). A similar epithelial replacement occurs at approximately 3 weeks of age in piglets, where differentiation to vacuolated enterocytes has been shown to be dependent on epithelial migration time (Moon, Kohler and Whipp, 1973). Reduced migration time in piglets older than 3 weeks is insufficient for ileal enterocytes to mature into neonatal vacuolated absorptive cells (Moon, 1983). Hence, if differentiation of enterocytes in the rat ileum has a similar dependency on migration time, this could explain the transient loss of vacuolated enterocytes from atrophic ileal villi at 8 days after infection.

Reduced migration time in areas of severe villous atrophy may result in undifferentiated trophozoite stages being lost into the gut lumen when enterocytes are extruded at the villous tip. This may be one mechanism whereby the reproductive potential of the parasite is limited by the host in the absence of specific immunity. Work by Koldovsky *et al* (1966) indicates that the rate of cellular migration in the small intestine of the adult rat is approximately 4-5 times faster than that in the preweaned rat. This may prevent the establishment of large endogenous stage populations in immunologically naive adults. Whether similar mechanisms could operate in the large intestine is unknown. It is difficult to appreciate changes in epithelial migration rate on standard histological examination of the large intestine and there is little information regarding post weaning changes in rodents.

In studies of other host-parasite systems, Pout (1967) suggested that the life cycles of *Eimeria acervulina* in the chicken and *E. crandallis* in sheep were synchronized with the villous migration times of epithelial cells in their respective hosts. Furthermore, pre-gamont stages of *E. crandallis* stimulate

division of host cells and replicate synchronously with them (Gregory, Catchpole, Pittilo and Norton, 1987).

If epithelial cell migration time can influence the reproductive potential of coccidian parasites, then it would be useful to know if *Cryptosporidium* sporozoites have a predilection to infect immature villous or crypt cells. Similarly, it would be useful to investigate whether merozoites involved in cyclic asexual activity move to lower, more immature, cells on the villi to compensate for the effects of villous atrophy on epithelial migration time. If infection of host cells does not occur at random, then this implies that motile stages of *Cryptosporidium* are receptive to differences between host cells. Differences in cell membrane glycoproteins have been shown to reflect the developmental state of crypt and villous cells (Weiser, 1973). These glycoproteins may act as receptors allowing the parasite to differentiate between host cells.

Diarrhoea was observed in sucking rats after they had been infected with *Cryptosporidium* at 4 days of age (Chapter 3, Experiment 3.2). Infection of rats, previously unexposed to *Cryptosporidium*, close to and after weaning, did not induce clinical signs, an observation which paralleled the published age susceptibility of specific pathogen free lambs (Tzipori *et al*, 1981c). Although far fewer endogenous stages were observed in the older rats they responded to a primary infection with higher serum antibody titres than rats infected at 4 days of age. It was unclear whether this age-related resistance was innate or because older animals were able to overcome the infection with a more effective specific immune response.

Resistance to *Cryptosporidium* is age-dependent. It has been shown that while athymic mice suffered prolonged oocyst shedding after infection at 4-6 days of age, their susceptibility was greatly reduced when they were infected at 42 days of age (Sherwood *et al*, 1982; Heine *et al*, 1984a). Antibody responses

were not measured in those studies and the involvement of specific immunity was not eliminated since nude mice have been shown to react normally to thymus-independent antigens such as bacterial lipopolysaccharide (Manning, Reed and Jutila, 1972). Nude mice have normal serum levels of IgM but reduced IgG and IgA levels (Pantelouris, 1978; Mink, Radl, van den Berg, Haaijman, Van Zweiten and Benner, 1980).

In addition to studying the nature and role of any specific immunity, attention should also be given to the possible involvement of non-specific mechanisms. Clinical cryptosporidiosis in domestic animals usually occurs during the first 4 weeks of life (Tzipori, 1988). Hence, attention could be focused on those parameters of the gastrointestinal tract known to change with age and which could influence the manifestation of the disease. An example, already discussed, would be the influence of a change in the enterocyte migration rate with age. Information is lacking on whether the properties of intestinal surface mucus change after weaning and if so, how this might influence motile stages of the parasite.

This study agreed with those of others (Sherwood *et al*, 1982; Current and Reese, 1986) in that enteric cryptosporidiosis in mice was asymptomatic and infection of the small intestine was predominantly in the ileum. Investigation of excystation (Chapter 3, Experiment 3.3) demonstrated that the distribution of infection and subsequent pathology could depend on the physiochemical status of the gut contents. Results showed that in the mouse small intestine, excystation conditions were more favourable in the distal segments. Experiments designed to duplicate, *in vitro*, the pH changes to which oocysts would be exposed in passage through the mouse stomach and distal small intestine elucidated two important excystation stimuli: (1) a primary period of incubation (37^{0}C) at a pH between 3.6-4.9 which in itself did not trigger excystation and (2) a subsequent pH shift from 4.9 to between 7.6 and 8.4 which triggered rapid

excystation especially in the presence of 7-deoxycholic acid. While bile salts are known to enhance excystation in some species of *Eimeria* (Ryley, 1973), the importance of a shift in pH, to the author's knowledge, has not been reported. The rapid and high percentage excystation (greater than 90% after 20 minutes incubation), achieved using the technique outlined in this study, greatly facilitated the use of *Cryptosporidium* sporozoites in both *in vitro* and *in vivo* investigations.

The distribution of infection in cryptosporidiosis may also be influenced by trapping of oocysts and sporozoites in small intestinal surface mucus. In infant rats oocysts and sporozoites appeared to be trapped in surface mucus along the entire length of the small intestine whereas in mice, this occurred only in the ileum (Chapter 3, Experiment 3.3). These observations agreed with the subsequent distribution of endogenous stages found in these species.

The present study provides the first assessment of the interaction between *Cryptosporidium* sporozoites, specific IgA and intestinal surface mucus. By 4 days after infection of mice with *Cryptosporidium* oocysts, the amount of surface mucus in the distal ileum was shown to have increased in comparison to age-matched uninfected controls. Surface mucus may be important to the parasite in providing a suitable medium for spread of motile endogenous stages and to the host in terms of its ability to modify the viscoelastic properties of the mucus during an inflammatory response.

Colonic mucus from an uninfected control gnotobiotic lamb agglutinated *Cryptosporidium* sporozoites. This agglutination was considered non-specific because it was not associated with detectable specific antibody. After gel filtration of this mucus, the agglutination was associated with hexose-rich fractions which suggested that mucus glycoproteins may have been involved. Purification of the mucus glycoprotein was not

undertaken in the present study but may be useful in further work. This work could follow the line of investigation established in studies of *Entamoeba histolytica*, where colonic mucin glycoproteins have been shown to act as an important host defence by binding to the parasite's adherence lectin, thus preventing amoebic attachment to and cytolysis of host epithelial cells (Leitch, Dickey, Udezulu and Bailey, 1985; Ravdin, John, Johnston, Innes and Guerrant, 1985; Chadee, Petri, Innes and Ravdin, 1987; Petri, Smith, Schlesinger, Murphy and Ravdin, 1987). However, when assessing the function of mucus, it is important to consider the influence of its other constituents. The characteristics of the final mucus product depend not only on interactions between glycoproteins but may also include interactions with other proteins and nucleic acids (Forstner, Wesley and Forstner, 1982).

Colonic mucus from a *Cryptosporidium* infected lamb was shown to have immunologically-specific sporozoite agglutinating activity in addition to the non-specific activity described above. The former was associated with mucus fractions which contained sporozoite-specific IgA. This study showed that the rise in faecal IgA titres between days 8 and 16 after infection coincided with the fall in oocyst output by infected lambs. Specific IgA, present in intestinal secretions, may block attachment receptors on motile stages of *Cryptosporidium*. Another role for IgA may be in increasing mucus viscosity thereby hindering the progress of motile stages. Both IgA and albumin have been shown to increase the viscosity of pig and dog gastric mucin glycoproteins (Forstner *et al* 1982; Murty, Sarosiek, Slomiany and Slomiany, 1984). This effect on viscosity might be expected with increasing amounts of albumin and IgA present on inflamed intestinal surfaces.

Inhibition of sporozoite penetration of host cells by IgA has also been suggested in studies of *Eimeria tenella* infection in chickens (Davis *et al*, 1978; Davis and Porter, 1979). Similarly,

expulsion of *Giardia muris* by mice was associated with the appearance and increasing levels of parasite-specific IgA in intestinal secretions (Snider and Underdown, 1986). Whereas crude caecal and colonic mucus from normal mice were without effect on the sporozoites of the coccidian parasite *Eimeria falciformis*, immune mucus caused them to agglutinate; immune mucus, unlike that from normal mice, contained sporozoite-specific IgA but not IgG or IgM as detected by an immunofluorescent antibody test (Douglass and Speer, 1985).

Using lambs as experimental animals, the present study provides the first detailed analysis of serum and secreted antibody responses in relation to the kinetics of *Cryptosporidium* oocyst shedding. Although the presence of serum antibody has been used in this and other studies (Tzipori and Campbell, 1981; Ungar *et al*, 1986; Casemore, 1987) as an indicator of the host immune response to *Cryptosporidium* infection, its functional significance remains uncertain. IgA and IgM antibodies to sporozoites were detected in sera and intestinal secretions of infected lambs (Chapter 4). Immunoblot analysis showed that IgA and IgM recognized numerous oocyst antigens over the same molecular weight range. Six antigens, with estimated molecular weights of 180(I), 93(II), 67(III), 47(IV), 23(V) and between 12.3 and 17.2(VI)Kd., were features on immunoblots developed with these antibodies. Titres of IgG rose slowly during the oocyst shedding period but, in comparison to IgM and IgA, it was found only in the serum and on immunoblot analysis, recognised relatively few oocyst antigens.

The relative numbers of IgM-, IgA- or IgG-producing immunocytes in the intestinal lamina propria of lambs were not investigated in this study. Studies of local antibody synthesis in the alimentary tracts of normal calves (Porter *et al*, 1972; Allen and Porter, 1975) and lambs (Husband and McDowell, 1975, 1978; Larsen and Landsverk, 1986) have indicated that IgM- and IgA-producing immunocytes are more numerous than IgG cells

during the first weeks of life. Production of specific IgG may have depended upon dissemination of antigens to lymphoid tissues beyond the intestine. The relatively few oocyst antigens recognised by IgG, in comparison to IgM and IgA, may reflect the restricted exposure of non-gut associated lymphoid tissue to *Cryptosporidium* antigens in young ruminants.

The results described in Chapters 4 and 5 indicated that, in lambs and probably other young ruminants, IgM and IgA were the major immunoglobulins available for activity against endogenous stages of the parasite attached to or present in mucus above host enterocytes. Both antibodies may act by agglutinating and hindering attachment of motile stages of the parasite. If complement proteins were present in serum exudates resulting from enteritis, then IgM may be able to participate in complement-mediated parasite destruction.

With regard to serum exudation into the bowel lumen, an interesting finding was that sporozoites incubated in either unheated hyperimmune or non-immune lamb sera were rapidly lysed and fragmented. That this lytic effect was greatly reduced after heat inactivation suggested that it was complement-mediated. Abundant specific antibody would have been available in the hyperimmune sera for participation in activation of the classical complement pathway. However, sporozoite lysis proceeded more rapidly in unheated sera from *Cryptosporidium* free lambs with no detectable specific antibody. If complement was involved in sporozoite lysis in these sera, then activation via the alternate pathway may have occurred.

Titration showed that the lytic capacity of unheated sera was greatest in those from uninfected gnotobiotic control lambs. Restoration of the lytic effect in inactivated sera by addition of fresh normal serum, as a complement source, would not have clarified the involvement of complement in view of the lytic activity in non-immune sera. Testing fractions of sera producing

non-specific sporozoite lysis (not associated with detectable specific antibody) may be useful in determining the molecular weight and properties of the active component(s).

Sporozoites of *Eimeria tenella* and merozoites of *E. bovis* have been observed to degenerate and/or lyse in the presence of both unheated immune and non-immune chicken and calf sera respectively (Long, Rose and Pierce, 1963; Andersen *et al*, 1965; Burns and Challey, 1965). The nature of the degeneration was similar to that described in the present study for *Cryptosporidium* sporozoites.

Any evaluation of lytic activity in immunity to cryptosporidiosis must consider the capacity of normal sera to lyse sporozoites and whether the serum component(s) responsible reach the bowel lumen. The latter process may be a pre-requisite for lytic activity since evidence for active penetration of *Cryptosporidium* sporozoites or other endogenous stages beyond the intestinal epithelium is lacking. The demonstration of lytic activity in the sera of 5-day old gnotobiotic lambs and a 28-day old colostrum-deprived conventional lamb which were *Cryptosporidium* free has shown that this capacity was not dependent on active or passive immunity.

The capacity of *Cryptosporidium* sporozoites to infect infant rats was greatly reduced after incubation with either heat-inactivated hyperimmune or non-immune lamb sera. This effect was demonstrated by complete neutralization without detection of endogenous stages in the gut lining. It was also demonstrated that sporozoite neutralization by hyperimmune lamb serum was associated with parasite-specific IgG which had been separated by affinity chromatography. However, removing total IgG from this hyperimmune serum did not eliminate its capacity to reduce sporozoite infectivity. The remaining activity could have been due to the unidentified heat-stable, non-dialysable component(s) responsible for the activity in uninfected gnotobiotic lamb serum.

The current study on the one hand supports the work of Riggs and Perryman (1987), in which hyperimmune bovine serum to sporozoites neutralized their capacity to infect sucking mice, but on the other hand differs in that sporozoite neutralization was not detected by these authors as a feature of pre-immunization serum. Whether sporozoite neutralization in their study was associated with parasite-specific IgG in the hyperimmune bovine serum or the immunization procedure had induced a non-specific neutralizing activity was not investigated.

Two further aspects of the work by Riggs and Perryman (1987) may have affected their results by accentuating any differences between immune and non-immune sera. First, sporozoites were subjected to prolonged excystation and isolation procedures (at least 1.5 hours) followed by interim storage at 4^{0}C prior to incubation in test serum at 37^{0}C. Approximately 85% of inoculated sporozoites were judged to be viable, as determined with fluorescein diacetate and fluorescence microscopy. Thus, the infectivity of their sporozoites may have been reduced in comparison to those used in the present study where excystation was a rapid process requiring only 30 minutes and was followed directly by incubation in test serum. Secondly, all mice were killed 4 days after inoculation of sporozoites and examined histologically for evidence of infection. As shown with mice and rats in the current study, examination at 3 and 5 days after inoculation and possibly longer is necessary in order to minimize the chances of overlooking small numbers of endogenous stages in a developing infection.

Reduced sporozoite infectivity associated with parasite-specific IgG may apply only within the circumstances defined by experiments in the current study, however it could have important implications for passive protection of young ruminants. Published information is lacking on whether young

ruminants sucking dams which were locally immunized are afforded protection against cryptosporidiosis. Local immunization of the ruminant mammary gland substantially increases the level of IgA in milk but IgG is still the most important isotype (Watson and Lascelles, 1973). Data available from studies in mice suggested that lacteal immunity provided little or no protection against cryptosporidiosis in this species (Moon *et al*, 1988). The effectiveness of lacteal immunity against cryptosporidiosis may vary between species and studies are needed to clarify this point.

Chapter 3 (Experiment 3.4) described a method whereby ileal mucus washings from *Cryptosporidium* infected infant mice could be used as a rich source of merozoites. This was the first time a method had been described which enabled collection of large numbers of *Cryptosporidium* merozoites. The method may prove useful in obtaining merozoite messenger RNA in studies of immunity requiring production of specific antigens using molecular biology methods. In addition, studies which currently rely on sporozoites to investigate potentially useful therapeutic agents and host immune mechanisms could now use merozoites to further validate their findings.

The present study provided the first comparative analysis of *Cryptosporidium* oocyst and merozoite antigens. When hyperimmune rabbit sera, raised against oocyst antigens, were reacted with blots of merozoite antigens, the reaction covered a similar molecular weight range to that seen with oocyst antigen and many bands appeared to be shared. Similarly, most merozoite antigens recognized by IgA, from intestinal secretions of convalescent lambs, appeared in a molecular weight range from 66-180Kd.,as did most of the oocyst antigens recognized by this antibody. However, a 23Kd. antigen which was detected on merozoite blots using hyperimmune rabbit sera was not detected by IgA. In addition, several oocyst antigens between 45 and 66Kd., detected by IgA, were not detected by this antibody on merozoite blots.

These antigenic differences may be useful in determining which stage of the parasite's life cycle is most concerned in the induction of protective immunity.

It would be interesting to know if infection with merozoites protected against subsequent challenge with sporozoites. This type of experiment has yielded valuable information in studies of *Eimeria* spp. (Rose, 1967b; Rose and Hesketh, 1976; McDonald *et al*, 1988). While most domestic and laboratory animal species are susceptible to *Cryptosporidium* infection as infants, by the time a primary infection has subsided, interpreting the results of a challenge infection may be difficult due to the onset of age-related resistance.

Studies with athymic rodents (Mesfin and Bellamy, 1979; Rose, Ogilvie, Hesketh and Festing, 1979) and attempts at protection by adoptive transfer of immune cells (Liburd, Armstrong and Mahrt, 1973; Rose *et al*, 1988) have indicated that immunity against coccidian parasites depends on cell-mediated responses. Cell-mediated immunity was not investigated in the current study but the inbred Lister rat model could be used for adoptive cell transfer studies. Cryptosporidiosis in the Lister rat has been described in terms of clinical signs, age-susceptibility and the kinetics and specificity of serum antibody response (Chapters 3, 4 and 5).

Studies with normal and athymic mice have provided evidence that the chronicity of *Giardia muris* infection in the latter results from lack of intestinal trophozoite-specific IgA in these animals (Heyworth, 1986; Heyworth, Kung and Caplin, 1988). These and experiments in the current study have highlighted the need to interpret, in investigations of enteric protozoan infections, the possible involvement of cell-mediated immune mechanisms alongside the kinetics of secretory antibody response as well as that in serum. The serum response may not reflect the concentration or isotype of antibody available at the site of

intestinal infection thus influencing any correlation between antibody titre and protection.

Neither the sequence, consistency nor prominence of the antigens recognized on immunoblots carried out with convalescent antibody in the present study enabled their categorization in order of importance to any possible antibody-mediated immunity. Electroelution of antigens separated on polyacrylamide gel and testing of specific antibody raised to them in *in vivo* or *in vitro* systems may be required for this determination. By chance or contrivance of the parasite, the host may not be exposed to certain protective antigens. Hence, the immunogenic properties of those antigens detected by hyperimmune serum but not convalescent serum should also be examined.

In conclusion, the current study has elucidated four mechanisms which could be important to the host in overcoming *Cryptosporidium* infection:

1. Loss of undifferentiated trophozoite stages sloughed with host cells from the villous tip. This may occur where reduced enterocyte migration time results from villous atrophy and crypt hyperplasia.

2. Non-specific (not associated with specific antibody) agglutination of motile endogenous stages in normal mucus preventing their access to epithelial cells. The increased surface mucus found during infection may enhance non-specific agglutination.

3. Agglutination of sporozoites by parasite-specific IgA in intestinal secretions. Merozoites are likely to be agglutinated by IgA in view of the numerous antigens they share with sporozoites. IgM (also found in intestinal secretions) may complement IgA agglutination and together these antibodies may block the attachment receptor sites on motile stages.

4. Non-specific reduction in the infectivity of motile asexual stages resulting from injury caused by heat-stable, non-dialysable components in normal serum. These unidentified components may gain access to the bowel lumen in inflammatory exudates subsequent to mucosal injury by the parasite.

BIBLIOGRAPHY

AL SALAMI, M., SIMPSON-MORGAN, M.W. and MORRIS, B. (1985). Haemopoiesis and the development of immunological reactivity in the sheep foetus. In: Immunology of the sheep (ed. B. Morris and M. Miyasaka). pp 19-36. Switzerland: F. Hoffmann-La Roche and Co.

ALLEN, A., BELL, A., MANTLE, M. and PEARSON, J.P. (1982). The structure and physiology of gastrointestinal mucus. In: Mucus in Health and Disease-II (ed. E.N. Chantler, J.B. Elder and M. Elstein), pp 115-133. London: Plenum Press

ALLEN, W.D. and PORTER, P. (1975). Localization of immunoglobulins in intestinal mucosa and the production of secretory antibodies in response to intraluminal administration of bacterial antigens in the preruminant calf. Clinical and Experimental Immunology, 21, 407-418.

ALPERT, G., BELL, L.M., KIRKPATRICK, C.E., BUDNICK, L.D., CAMPOS, J.M., FRIEDMAN, L.M. and PLOTKIN, S.A. (1986). Outbreak of cryptosporidiosis in a day-care centre. Pediatrics, 77, 152-157.

ANDERSEN, F.L., LOWDER, L.J., HAMMOND, D.M. and CARTER, P.B. (1965). Antibody production in experimental *Eimeria bovis* infections in calves. Experimental Parasitology, 16, 23-25.

ANDERSON, B.C. (1981). Patterns of shedding of cryptosporidial oocysts in Idaho calves. Journal of the American Veterinary Medical Association, 178, 982-984.

ANDERSON, B.C. (1982). Cryptosporidiosis in Idaho lambs: natural and experimental infections. Journal of the American Veterinary Medical Association, 181, 151-153.

ANDERSON, B.C. (1987). Abomasal cryptosporidiosis in cattle. Veterinary Pathology, 24, 235-238.

ANGUS, K.W. (1987). Cryptosporidiosis in domestic animals and humans. In Practice, 9, 47-49.

ANGUS, K.W., APPLEYARD, W.T., MENZIES, J.D., CAMPBELL,I. and SHERWOOD, D. (1982a). An outbreak of diarrhoea associated with cryptosporidiosis in naturally reared lambs. Veterinary Record, 110, 129-130.

ANGUS, K.W., TZIPORI, S. and GRAY, E.W. (1982b). Intestinal lesions in specific-pathogen-free lambs associated with *Cryptosporidium* from calves with diarrhoea. Veterinary Pathology, 19, 67-68.

ANGUS, K.W., HUTCHISON, G., CAMPBELL, I. and SNODGRASS, D.R. (1984). Prophylactic effects of anticoccidial drugs in experimental murine cryptosporidiosis. Veterinary Record, 114, 166-168.

ANGUS, K.W., HUTCHISON, G. and MUNRO, H.M.C. (1985). Infectivity of a strain of *Cryptosporidium* found in the guinea-pig (*Cavia porcellus*) for guinea-pigs, mice and lambs. Journal of Comparative Pathology, 95, 151-165.

BABB, R.R., DIFFERDING, J.T. and TROLLOPE, M.L. (1982). Cryptosporidia in a healthy professional athlete. American Journal of Gastroenterology, 77, 833-834.

BESSER, T.E., McGUIRE, T.C. and GAY, C.C. (1987). The transfer of serum IgG_1 antibody into the gastrointestinal tract in newborn calves. Veterinary Immunology and Immunopathology, 17, 51-56.

BIRD, R.G. and SMITH, M.D. (1980). Cryptosporidiosis in man: parasite life cycle and fine structural pathology. Journal of Pathology, 132, 217-233.

BLACK, R.E. (1985). Global problems of acute diarrhoea in young children. In: Infectious Diarrhoea in the Young. Strategies for Control in Humans and Animals (ed. S. Tzipori), pp 3-8. Amsterdam: Elsevier Science Publishers B.V. (Biomedical Division). Excerpta Medica International Congress Series 674.

BLAGBURN, B.L. and CURRENT, W.L. (1983). Accidental infection of a researcher with human *Cryptosporidium*. Journal of Infectious Diseases, 148, 772-773.

BLEWETT, D.A. (1987). Cryptosporidiosis in red deer. In: The Management and Health of Farmed Deer (ed. H.W. Reid), pp 43-47. Kluwer Academic Publishers.

BLEWETT, D.A. (1988). Quantitative techniques in *Cryptosporidium* research. In: Cryptosporidiosis, Proceedings of the First International Workshop (ed. K.W. Angus and D.A. Blewett), p 85-95. Edinburgh: Dupli-quick Printers.

BORTHISTLE, B.K., KUBO, R.T., BROWN, W.R. and GREY, H.M. (1977). Studies on receptors for IgG on epithelial cells of the rat intestine. Journal of Immunology, 119, 471-475.

BOX, E.D., MARCHIONDO, A.A., DUSZYNSKI, D.W. and DAVIS, C.P. (1980). Ultrastructure of *Sarcocystis* sporocysts from passerine birds and opossums: comments on classification of the genus *Isospora*. Journal of Parasitology, 66, 68-74.

BRADBURY, J.E., BLACK, J.W. and WYLLIE, J.H. (1980). Stimulation of mucus output from rat colon *in vivo*. European Journal of Pharmarcology, 68, 417-425.

BULGIN, M.S., ANDERSON, B.C., WARD, A.C.S. and EVERMANN, J.F. (1982). Infectious agents associated with neonatal calf disease in southwestern Idaho and eastern Oregon. Journal of the American Veterinary Medical Association, 180, 1222-1226.

BURNS, W.C. and CHALLEY, J.R. (1965). Serum lysins in chickens infected with *Eimeria tenella*. Journal of Parasitology, 51, 660-668.

CAMPBELL, I., TZIPORI, S., HUTCHISON, G. and ANGUS, K.W. (1982). Effect of disinfectants on survival of *Cryptosporidium* oocysts. Veterinary Record, 111, 414-415.

CAMPBELL, P.N. and CURRENT, W.L. (1983). Demonstration of serum antibodies to *Cryptosporidium* sp. in normal and immunodeficient humans with confirmed infections. Journal of Clinical Microbiology, 18, 165-169.

CASEMORE, D.P. (1987). The antibody response to *Cryptosporidium*: development of a serological test and its use in a study of immunologically normal persons. Journal of Infection, 14, 125-134.

CHADEE, K., PETRI, Jr., W.A., INNES, D.J. and RAVDIN, J.I. (1987). Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. Journal of Clinical Investigation, 80, 1245-1254.

CHENG, H. and LEBLOND, C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine 1. Columnar cell. American Journal of Anatomy, 141, 461-480.

CHERMETTE, R., BOUFASSA, S., SOULE, C. TARNAU, C., COURDERC, O. and LENGRONNE, D. (1987). La cryptosporidiose équine: une parasitose méconnue. Bulletin Cereopa, 13 ème Journee d'Etude, 11 mars, Paris, 81-84.

CLAMP, J.R. (1977). The relationship between secretory immunoglobulin A and mucus. Biochemical Society Transactions, 5, 1579- 1581.

CLAMP, J. (1986). The role of mucus in human intestinal defence. In: Gut Defences in Clinical Practice (ed. M.S. Losowsky and R.V. Heatby). pp 83-94. Edinburgh: Churchill Livingstone.

CLARKE, R.M. and HARDY, R.N. (1969). An analysis of the mechanism of cessation of uptake of macromolecular substances by the intestine of the young rat (closure). Journal of Physiology, 204, 127-134.

CORNELL, R. and PADYKULA, H.A. (1969). A cytological study of intestinal absorption in the suckling rat. American Journal of Anatomy, 125, 291-316.

CRANE, M.S.J., MURRAY, P.K., GNOZZIO, M.J. and MacDONALD, T.T. (1988). Passive protection of chickens against *Eimeria tenella* infection by monoclonal antibody. Infection and Immunity, 56, 972-976.

CRIPPS, A.W., HUSBAND, A.J. and LASCELLES, A.K. (1974). The origin of immunoglobulins in intestinal secretion of sheep. Australian Journal of Experimental Biology and Medical Science, 52, 711-716.

CROSS, R.F. and MOORHEAD, P.D. (1983). A rapid method for staining cryptosporidia in faecal smears. Proceedings: 26th Annual Meeting, American Association of Veterinary Laboratory Diagnosticians, 229-230.

CURRENT, W.L. and HAYNES, T.B. (1984). Complete development of *Cryptosporidium* in cell culture. Science, 224, 603-605.

CURRENT, W.L. and LONG, P.L. (1983). Development of human and calf *Cryptosporidium* in chicken embryos. Journal of Infectious Diseases, 148, 1108-1113.

CURRENT, W.L. and REESE, N.C. (1986). A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. Journal of Protozoology, 33, 98-108.

CURRENT, W.L., UPTON, S.J. and HAYNES, T.B. (1986). The life-cycle of *Cryptosporidium baileyi* n.sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. Journal of Protozoology, 33, 289-296.

DAVIS, P.J., PARRY, S.H. and PORTER, P. (1978). The role of secretory IgA in anti-coccidial immunity in the chicken. Immunology, 34, 879-888.

DAVIS, P.J. and PORTER, P. (1979). A mechanism for secretory IgA-mediated inhibition of the cell penetration and intracellular development of *Eimeria tenella*. Immunology, 36, 471-477.

DE VISSER, N.A.P.C., BREUKINK, H.J., VAN ZIJDERVELD, F.G. and DE LEEUW, P.W. (1987). Enteric infections in veal calves: a longitudinal study on four veal calf units. Veterinary Quarterly, 9, 289-296.

DOBSON, C. (1966). Precipitating antibodies in extracts from the mucosa and tunica muscularis of the alimentary tract of sheep infected with *Oesophagostomum columbianum*. Journal of Parasitology, 52, 1037-1038.

DOUGLASS , T.G. and SPEER, C.A. (1985). Effects of intestinal contents from normal and immunized mice on sporozoites of *Eimeria falciformis*. Journal of Protozoology, 32, 156-163.

DRURY, R.A.B. and WALLINGTON, E.A. (1967). In: Carleton's Histological Technique 4th edn., (a) Fixation p 44; (b) General staining procedures p 129; (c) Protozoa p 362 . Oxford University Press.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS P.A. and SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350-356.

EBERSOLE, J.L., SMITH, D.J. and TAUBMAN, M.A. (1985). Secretory immune responses in ageing rats. Immunology, 56, 345-350.

EBERSOLE, J.L., TAUBMAN, M.A. and SMITH, D.J. (1979). The effect of neonatal thymectomy on the level of salivary and serum immunoglobulin in rats. Immunology, 36, 649-657.

EBERSOLE, J.L., TAUBMAN, M.A., SMITH, D.J. and FREY, D.E. (1982). Effect of neonatal thymectomy on immune responses of rats to *Streptococcus mutans*. Infection and Immunity, 37, 993-1000.

ENDERS, G.A., BALLHAUS, S. and BRENDAL, W. (1988). The influence of Peyer's patches on the organ-specific distribution of IgA plasma cells. Immunology, 63, 411-414.

FAHEY, K.J. and MORRIS B. (1978). Humoral immune responses in foetal sheep. Immunology, 35, 651-661.

FAYER, R. and LEEK, R.G. (1984). The effects of reducing conditions, medium, pH, temperature and time on *in vitro* excystation of *Cryptosporidium*. Journal of protozoology, 31, 567-569.

FISHER, E.W., MARTINEZ, A.A., TRAININ, Z. and MEIROM, R. (1975). Studies of neonatal calf diarrhoea . II. Serum and faecal immune globulins in enteric colibacillosis. British Veterinary Journal, 131, 402-415.

FORD, J.E., PORTER, J.W.G., THOMPSON, S.Y., TOOTHILL, J. and EDWARDS-WEBB, J. (1969). Effects of ultra-high-temperature (UHT) processing and of subseguent storage on the vitamin content of milk. Journal of Dairy Research, 36, 447-454.

FORSTNER, G., WESLEY, A. and FORSTNER, J. (1982). Clinical aspects of gastrointestinal mucus. In: Mucus in Health and Disease-II (ed. E.N. Chantler, J.B. Elder and M. Elstein), pp 199-224. London: Plenum Press.

GIBSON, J.A., HILL, M.W.M. and HUBER, M.J. (1983). Cryptosporidiosis in Arabian foals with severe combined immunodeficiency. Australian Veterinary Journal, 60, 378-379.

GOEBEL, E. and BRAENDLER, U. (1982). Ultrastructure of microgametogenesis, microgametes and gametogamy of *Cryptosporidium* sp. in the small intestine of mice. Protistologica, 18, 331-334.

GRABAR, P. and WILLIAMS, C.A. (1953). Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Application au sérum sanguin. Biochimica et Biophysica acta, 10, 193-194. Cited from Bibi Wallenborg and Ulla-Birgitta Andersson (1978), LKB Application Note 249 (Sweden).

GREGORY, M.W., CATCHPOLE, J., PITTILO, R.M. and NORTON, C.C. (1987). How to harmonize with your host - synchronous division in the coccidia. The British Society for Parasitology; Spring Meeting 1987; Session 10A Abstract 151.

HAAIJMAN, J.J., SLINGERLAND-TEUNISSEN, J., VAN OUDENAREN, A., MINK, J.G. and BENNER, R. (1980). Kinetics of recovery of serum Ig levels and of cytoplasmic Ig positive cells in various lymphoid organs of nude mice after thymus transplantation. Immunology, 41, 279-287.

HALLIDAY, R. (1965). The transfer of antibodies from ewes to their lambs. Journal of Immunology, 95, 510-511.

HAMPTON, J.C. and ROSARIO,B. (1966). The attachment of protozoan parasites to intestinal epithelial cells of the mouse. Journal of Parasitology, 52, 939-949.

HARDY, R.N. (1969). The influence of specific chemical factors in the solvent on the absorption of macromolecular substances from the small intestine of the new-born calf. Journal of Physiology, 204, 607-632.

HARP, J.A., WANNEMUEHLER, M.W., WOODMANSEE, D.B. and MOON, H.W. (1988). Susceptibility of germfree or antibiotic-treated adult mice to *Cryptosporidium parvum*. Infection and Immunity, 56, 2006-2010.

HEALY, P.J. (1977). Intestinal brush border and lysosomal enzymes and immunoglobulin absorption in the newly-born lamb. Australian Journal of Experimental Biology and Medical Science, 55, 327-337.

HEINE, J., MOON, H.W. and WOODMANSEE, D.B. (1984a). Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. Infection and Immunity, 43, 856-859.

HEINE, J., POHLENZ, J.F.L., MOON, H.W. and WOODE, G.N. (1984b). Enteric lesions and diarrhoea in gnotobiotic calves monoinfected with *Cryptosporidium* species. Journal of Infectious Diseases, 150, 768-775.

HENRIKSEN, Sv.Aa. and KROGH, H.V. (1985). Bovine cryptosporidiosis in Denmark. 1. Prevalence, age distribution and seasonal variation. Nordisk Veterinarmedicin, 34-41.

HEYWORTH, M.F. (1986). Antibody response to *Giardia muris* trophozoites in mouse intestine. Infection and Immunity, 52, 568-571.

HEYWORTH, M.F., KUNG, J.E. and CAPLIN, A.B. (1988). Enzyme-linked immunosorbant assay for *Giardia*-specific IgA in mouse intestinal secretions. Parasite Immunology, 10, 713-717.

HOROWITZ, M.I. (1977). Gastrointestinal glycoproteins . In: The Glycoconjugates, Vol. 1 Mammalian Glycoproteins and Glycolipids (ed. M.I. Horowitz and W. Pigman), pp 189-209. Academic Press (Harcourt Brace Jovanovich, publishers).

HUMMEL, K.P. (1935). The structure and development of the lymphatic tissue in the intestine of the albino rat. American Journal of Anatomy, 57, 351-377.

HUNTLEY, J.F., NEWLANDS, G.F.J., MILLER, H.R.P., McLAUCHLAN, M., ROSE, M.E. and HESKETH, P. (1985). Systemic release of mucosal mast cell protease during infection with the intestinal protozoan parasite, *Eimeria nieschulzi*. Studies in normal and nude rats. Parasite Immunology, 7, 489-501.

HUSBAND, A.J. and LASCELLES, A.K. (1974). The origin of antibody in intestinal secretion of sheep. Australian Journal of Experimental Biology and Medical Science, 52, 791-799.

HUSBAND, A.J. and McDOWELL, G.H. (1975). Local and systemic immune responses following oral immunization of foetal lambs. Immunology, 29, 1019-1028.

HUSBAND, A.J. and McDOWELL, G.H. (1978). Immunity to experimental enteritis in lambs vaccinated prenatally. Research in Veterinary Science, 25, 343-349.

ISEKI, M. (1979). *Cryptosporidium felis* sp.n. (Protozoa: Emeriorina) from the domestic cat. Japanese Journal of Parasitology, 28, 285-307.

ITAKURA, C., NAKAMURA, H., UMEMURA, T. and GORYO, M. (1985). Ultrastructure of cryptosporidial life cycle in chicken host cells. Avian Pathology, 14, 237-249.

JAFFE, H.W., BREGMAN, D.J. and SELIK, R.M.(1983). Acquired immune deficiency syndrome in the United States: the first 1000 cases. Journal of Infectious Diseases, 148, 339-345.

JERRETT, I.V. and SNODGRASS, D.R. (1981). Cryptosporidia associated with outbreaks of neonatal calf diarrhoea. Australian Veterinary Journal, 57, 434-435.

JUBB, K.V.F., KENNEDY, P.C. and PALMER, N. (1985). In: Pathology of Domestic Animals. 3rd edn., Vol. 2. (a) p 200; (b) pp 120-122; (c) pp 68-69; (d) p 72. Academic Press, Inc. (Harcourt Brace Jovanovich, publishers).

KAPLAN, B.S., UNI, S., AIKAWA, M. and MAHMOUD, A.A.F. (1985). Effector mechanism of host resistance in murine giardiasis: specific IgG and IgA cell-mediated toxicity. Journal of Immunology, 134, 1975-1981.

KLAUS, G.G.B., BENNET, A. and JONES, E.W. (1969). A quantitative study of the transfer of colostral immunoglobulins to the newborn calf. Immunology, 16, 293-295.

KLESIUS, P.H., HAYNES, T.B. and MALO, L.K. (1986). Infectivity of *Cryptosporidium* sp. isolated from wild mice for calves and mice. Journal of the American Veterinary Medical Association, 189, 192-193.

KOLDOVSKY, O., SUNSHINE, P. and KRETCHMER, N. (1966). Cellular migration of intestinal epithelia in suckling and weaned rats. Nature, 212, 1389-1390.

KYHSE-ANDERSEN, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. Journal of Biochemical and Biophysical Methods, 10, 203-209.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-684. Cited from: HAMES, B.D. (1981). An introduction to polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins, A Practical Approach (ed. B.D. Hames and D. Rickwood), pp 1-86. Oxford: IRL Press, Limited.

LANDSVERK, T. (1987). Cryptosporidiosis and the follicle -associated epithelium over the ileal Peyer's patch in calves. Research in Veterinary Science, 42, 299-306.

LARSEN, H.J. and LANDSVERK, T. (1986). Distribution of T and B lymphocytes in jejunal and ileocaecal Peyer's patches of lambs. Research in Veterinary Science, 40, 105-111.

LASCELLES, A.K., BEH, K.J., KERLIN, R.L., WATSON, D.L. and MUKKUR, T.K.S. (1985). The generation of antibody responses of different isotype specificity in relation to mucosal defence in ruminants. In: Immunology of the Sheep (ed. B. Morris and M. Miyasaka). pp 410-435. Switzerland: F. Hoffmann - La Roche and Co.

LAZO, A., BARRIGA, O.O., REDMAN, D.R. and BECH-NIELSEN, S. (1986). Identification by transfer blot of antigens reactive in the enzyme-linked immunosorbent assay (ELISA) in rabbits immunized and a calf infected with *Cryptosporidium* sp. Veterinary Parasitology, 21, 151-163.

LECCE, J.G. and MORGAN, D.O. (1962). Effect of dietary regimen on cessation of intestinal absorption of large molecules (closure) in the neonatal pig and lamb. Journal of Nutrition, 78, 263-268.

LEITCH, G.J., DICKEY, A.D., UDEZULU, I.A. and BAILEY, G.B. (1985). *Entamoeba histolytica* trophozoites in the lumen and mucus blanket of rat colons studied *in vivo*. Infection and Immunity, 47, 68-73.

LEVINE, N.D. (1973). Introduction, history and taxonomy. In: The Coccidia: *Eimeria, Isospora, Toxoplasma* and Related Genera. (ed. D.M. Hammond and P.L. Long), pp 1-23. University Park Press, Baltimore and Butterworth and Co. (Publishers) Ltd., London.

LEVINE, N.D. (1980). Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. Journal of Parasitology, 66, 830-834.

LIBURD, E.M., ARMSTRONG, W.D. and MAHRT, J.L. (1973). Immunity to the protozoan parasite *Eimeria nieschulzi* in inbred CD-F rats. Cellular Immunology, 7, 444-452.

LIEBLER, E.M., POHLENZ, J.F. and WOODMANSEE, D.B. (1986). Experimental intrauterine infection of adult BALB/C mice with *Cryptosporidium* sp. . Infection and Immunity, 54, 255-259.

LINDSAY, D.S., BLAGBURN, D.L. and HOERR F.J. (1987). Experimentally induced infections in turkeys with *Cryptosporidium baileyi* isolated from chickens. American Journal of Veterinary Research, 48, 104-108.

LINDSAY, D.S., BLAGBURN, B.L., SUNDERMANN, C.A., HOERR, F.J. and ERNEST, J.A. (1986). Experimental *Cryptosporidium* infections in chickens: oocyst structure and tissue specificity. American Journal of Veterinary Research, 47, 876-879.

LINKS, I.J. (1982). Cryptosporidial infection of piglets. Australian Veterinary Journal, 58, 60-62.

LONG, P.L. and MILLARD, B.J. (1968). *Eimeria*: Effect of meticlorpindol and methyl benzoquate on endogenous stages in the chicken. Experimental Parasitology, 23, 331-338.

LONG, P.L., ROSE, M.E. and PIERCE, A.E. (1963). Effects of fowl sera on some stages in the life cycle of *Eimeria tenella*. Experimental Parasitology, 14, 210-217.

LUFT, B.J., PAYNE, D., WOODMANSEE, D. and KIM, C.W. (1987). Characterization of the *Cryptosporidium* antigens from sporulated oocysts of *Cryptosporidium parvum*. Infection and Immunity, 55, 2436-2441.

MACDOUGALL, D.F. and MULLIGAN, W. (1969). The distribution and metabolism of fast IgG immunoglobulin in the neonatal calf. Journal of Physiology, 201, 77P-78P. (Proceedings of the Physiological Society).

MALICK, L.E. and WILSON, R.B. (1975). Modified thiocarbohydrazide procedure for scanning electron microscopy: routine use for normal, pathological or experimental tissues. Stain Technology, 50, 265-269.

MANNING, J.K., REED, N.D. and JUTILA, J.W. (1972). Antibody response to *Escherichia coli* lipopolysaccharide and type III pneumococcal polysaccharide by congenitally thymusless (nude) mice. Journal of Immunology, 108, 1470-1471.

MANTLE, M. and ALLEN, A. (1981). Isolation and characterization of the native glycoprotein from pig small intestinal mucus. Biochemical Journal, 195, 267-275.

MARCIAL, M.A. and MADARA, J.L. (1986). *Cryptosporidium*: cellular localization, structural analysis of absorptive cell-parasite membrane-membrane interactions in guinea pigs and suggestion of protozoan transport by M cells. Gastroenterology, 90, 583-594.

MARSH, C.L., MEBUS, C.A. and UNDERDAHL, N.R. (1969). Loss of serum proteins via the intestinal tract in calves with infectious diarrhoea. American Journal of Veterinary Research, 30, 163-166.

MARSHALL, T. and ALLEN ,A. (1977). The isolation of a high-molecular-weight glycoprotein from pig colonic mucus. Biochemical Society Transactions, 5, 436-439.

McDERMOTT, J.J. and STAUBER, L.A. (1954). Preparation and agglutination of merozoite suspensions of the chicken coccidian, *Eimeria tenella*. Journal of Parasitology, 40, 39A.

McDONALD, V., WISHER, M.H., ROSE, M.E. and JEFFERS, T.K. (1988). *Eimeria tenella*: immunological diversity between asexual generations. Parasite Immunology, 10, 649-660.

McNULTY, M.S. (1985). Enteric viral infections in young animals. In: Infectious Diarrhoea in the Young. Strategies for Control in Humans and Animals (ed. S. Tzipori), pp 231-239. Amsterdam: Elsevier Science Publishers B.V. (Biomedical Division). Excerpta Medica International Congress Series 674.

MEAD, J.R., ARROWOOD, M.J., CURRENT, W.L. and STERLING, C.R. (1988). Field inversion gel electrophoretic separation of *Cryptosporidium* spp. chromosome-sized DNA. Journal of Parasitology, 74, 366-369.

MEISEL, J.L., PERERA, D.R., MELIGRO, C. and ROBIN, C.E. (1976). Overwhelming watery diarrhoea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology, 70, 592-598.

MESFIN, G.M. and BELLAMY, J.E.C. (1979). Thymic dependence of immunity to *Eimeria falciformis* var. *pragensis* in mice. Infection and Immunity, 23, 460-464.

MINK, J.G., RADL, J., VAN DEN BERG, P., HAAIJMAN, J.J., VAN ZWIETEN, M.J., and BENNER, R. (1980). Serum immunoglobulins in nude mice and their heterozygous littermates during ageing. Immunology, 40, 539.

MOCKETT, A.P.A. and ROSE, M.E. (1986). Immune responses to *Eimeria*: quantification of antibody isotypes to *Eimeria tenella* in chicken serum and bile by means of the ELISA. Parasite Immunology, 8, 481-489.

MOON H.W. (1983). Intestine. In: Cell Pathology. 2nd edn. (ed. N.F. Cheville), pp 503-529. Ames, Iowa: The Iowa State University Press.

MOON, H.W. and BEMRICK, W.J. (1981). Faecal transmission of calf cryptosporidia between calves and pigs. Veterinary Pathology, 18, 248-255.

MOON, H.W. and JOEL, D.D. (1975). Epithelial cell migration in the small intestine of sheep and calves. American Journal of Veterinary Research, 36, 187-189.

MOON, H.W., KOHLER, E.M., and WHIPP, S.C. (1973). Vacuolation: a function of cell age in porcine ileal absorptive cells. Laboratory Investigation, 28, 23-28.

MOON, H.W., McCLURKIN, A.W., ISAACSON, R.E., POHLENZ, J., SKARTVEDT, S.M., GILETTE, K.G. and BAETZ, A.L. (1978). Pathologic relationships of rotavirus, *Escherichia coli* and other agents in mixed infections in calves. Journal of the American Veterinary Medical Association, 173, 577-583.

MOON, H.W., SCHWARTZ, A., WELCH, M.J., McCANN, P.P. and RUNNELS, P.L. (1982a). Experimental faecal transmission of human cryptosporidia to pigs and attempted treatment with an ornithine decarboxylase inhibitor. Veterinary Pathology, 19, 700-707.

MOON, H.W., WOODE, G.N. and AHRENS, F.A. (1982b). Attempted chemoprophylaxis of cryptosporidiosis in calves. Veterinary Record, 110, 181.

MOON, H.W., WOODMANSEE, D.B., HARP,J.A., ABEL, S. and UNGAR, B.L.P. (1988). Lacteal immunity to enteric cryptosporidiosis in mice: immune dams do not protect their suckling pups. Infection and Immunity, 56, 649-653.

MORIN, M., LARIVIÈRE, S. and LALLIER, R. (1976). Pathological and microbiological observations made on spontaneous cases of acute neonatal calf diarrhoea. Canadian Journal of Comparative Medicine, 40, 228-240.

MORRIS, I.G. (1980). An immunofluoresence study of IgG receptors in rodent enterocytes. Immunology, 40, 273-275.

MURTY, V.L.N., SAROSIEK, J., SLOMIANY, A. and SLOMIANY, B.L. (1984). Effect of lipids and proteins on the viscosity of gastric mucus glycoprotein. Biochemical and Biophysical Research Communications, 121, 521-529.

NICHOLS, G. and THOM, B.T. (1984). Screening for *Cryptosporidium* in stools. Lancet, March 31, p 735.

O'DONOGHUE, P.J. (1985). *Cryptosporidium* infections in man, animals, birds and fish. Australian Veterinary Journal, 62, 253-258.

O'DONOGHUE, P.J., THAM, V.L., DE SARAM, W.G., PAULL, K.L. and McDERMOTT, S. (1987). *Cryptosporidium* infections in birds and mammals and attempted cross-transmission studies. Veterinary Parasitology, 26, 1-11.

OUCHTERLONY, Q. (1949). Antigen-antibody reactions in gels. II Factors determining the site of the precipitate. Arkiv för Kemi, 1, 43 43. Cited from Bibi Wallenborg and Ulla-Birgitta Andersson (1978), LKB Application Note 249 (Sweden).

PANCIERA, R.J., THOMASSEN, R.W. and GARNER, F.M. (1971). Cryptosporidial infection in a calf. Veterinary Pathology, 8, 479-484.

PANTELOURIS, E.M. (1978). Involution of the thymus and ageing. In: The Nude Mouse in Experimental and Clinical Research (ed. J. Fogh and B.C. Giovanella), p 51. New York: Academic Press.

PAPADOPOULOU, C., XYLOURI, E., MANTZIOS, G., SPYROPOULOS, G. and STOFOROS, S. (1988). Cryptosporidiosis in farm animals in Greece. In: Cryptosporidiosis, Proceedings of the First International Workshop. (ed. K.W. Angus and D.A. Blewett) p 123. Edinburgh: Dupli-quick Printers.

PEARSON, G.R. and LOGAN, E.F. (1983). Scanning and transmission electron microscopic observations on the host-parasite relationship in intestinal cryptosporidiosis of neonatal calves. Research in Veterinary Science, 34, 149-154.

PETRI, Jr., W.A., SMITH, R.D., SCHLESINGER, P.H., MURPHY, C.F. and RAVDIN, J.I. (1987). Isolation of the galactose-binding lectin that mediates the *in vitro* adherence of *Entamoeba histolytica*. Journal of Clinical Investigation, 80, 1238-1244.

POHJOLA, S. (1984). Negative staining method with nigrosin for the detection of cryptosporidial oocysts: a comparative study. Research in Veterinary Science, 36, 217-219.

POHLENZ, J., BEMRICK, W.J., MOON, H.W. and CHEVILLE, N.F. (1978a). Bovine cryptosporidiosis: a transmission and scanning electron microscopic study of some stages in the life cycle and of the host-parasite relationship. Veterinary Pathology, 15, 417-427.

POHLENZ, J., MOON, H.W., CHEVILLE, N.F. and BEMRICK, W.J. (1978b). Cryptosporidiosis as a probable factor in neonatal diarrhoea of calves. Journal of the American Veterinary Medical Association, 172, 452-457.

PORTER, P. (1972). Immunoglobulins in bovine mammary secretions. Quantitative changes in early lactation and absorption by the neonatal calf. Immunology, 23, 225-238.

PORTER, P., NOAKES, D.E. and ALLEN, W.D. (1970). Secretory IgA and antibodies to *Escherichia coli* in porcine colostrum and milk and their significance in the alimentary tract of the young pig. Immunology, 18, 245-257.

PORTER, P., NOAKES, D.E. and ALLEN, W.D. (1972). Intestinal secretion of immunoglobulins in the preruminant calf. Immunology, 23, 299-312.

POUT, D.D. (1967). Villous atrophy and coccidiosis. Nature, 213, 306-307.

QUIN, J.W., HUSBAND, A.J. and LASCELLES, A.K. (1975). The origin of the immunoglobulins in intestinal lymph of sheep. Australian Journal of Experimental Biology and Medical Science, 53, 205-214.

RADOSTITS, O.M. (1985). A veterinary clinician's perspective of diarrhoea in neonatal food-producing animals. In: Infectious Diarrhoea in the Young. Strategies for Control in Humans and Animals (ed. S. Tzipori), pp 9-18. Amsterdam: Elsevier Science Publishers B.V. (Biomedical Division). Excerpta Medica International Congress Series 674.

RAVDIN, J.J., JOHN, J.E., JOHNSTON, L.I., INNES, D.J. and GUERRANT, R.L. (1985). Adherence of *Entamoeba histolytica* trophozoites to rat and human colonic mucosa. Infection and Immunity, 48, 292-297.

REDUKER, D.W. and SPEER, C.A. (1985). Factors influencing excystation in *Cryptosporidium* oocysts from cattle. Journal of Parasitology, 71, 112-115.

REDUKER, D.W. and SPEER, C.A. (1986). Proteins and antigens of merozoites and sporozoites of *Eimeria bovis* (Apicomplexa). Journal of Parasitology, 72, 901-907.

REHG, J.E., HANCOCK, M.L. and WOODMANSEE, D.B. (1987). Characterization of cyclophosphamide-rat model of cryptosporidiosis. Infection and Immunity, 55, 2669-2674.

REINEMEYER, C.R., KLINE, R.C. and STAUFFER, G.D. (1984). Absence of *Cryptosporidium* oocysts in faeces of neonatal foals. Equine Veterinary Journal, 16, 217-218.

REYNOLDS, J. (1980). Gut-associated lymphoid tissues in lambs before and after birth. Monographs in Allergy, 16, 187-202.

REYNOLDS, J.D. (1985). The influence of antigen on Peyer's patches of sheep. In: Immunology of the Sheep (ed. B. Morris and M. Miyasaka), pp 216-231. Switzerland: F. Hoffmann - La Roche and Co.

REYNOLDS, D.J., MORGAN, J.H., CHANTER, N., JONES, P.W., BRIDGER, J.C., DEBNEY, T.G. and BUNCH, K.J. (1986). Microbiology of calf diarrhoea in southern Britain. Veterinary Record, 119, 34-39.

RIGGS, M.W. and PERRYMAN, L.E. (1987). Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infection and Immunity, 55, 2081- 2087.

RINDERKNECHT, H. (1962). Ultra-rapid fluorescent labelling of proteins. Nature, 193, 167-168.

RODEWALD, R. (1973). Intestinal transport of antibodies in the newborn rat. Journal of Cell Biology, 58, 189-211.

ROSE, M.E. (1967a). The influence of age of host on infection with *Eimeria tenella*. Journal of Parasitology, 53, 924-929.

ROSE, M.E. (1967b). Immunity to *Eimeria tenella* and *Eimeria necatrix* infections in the fowl. I. Influence of the site of infection and the stage of the parasite. II. Cross-protection. Parasitology, 57, 567-583.

ROSE, M.E. and HESKETH, P. (1976). Immunity to coccidiosis: stages of the life-cycle of *Eimeria maxima* which induce, and are affected by, the response of the host. Parasitology, 73, 25-37.

ROSE, M.E. and HESKETH, P. (1982). Coccidiosis: T-lymphocyte -dependent effects of infection with *Eimeria nieschulzi* in rats. Veterinary Immunology and Immunopathology, 3, 499-508.

ROSE, M.E. and LONG, P.L. (1971). Immunity to coccidiosis: protective effects of transferred serum and cells investigated in chick embryos infected with *Eimeria tenella*. Parasitology, 63, 299-313.

ROSE, M.E., OGILVIE, B.M., HESKETH, P. and FESTING, M.F.W. (1979). Failure of nude (athymic) rats to become resistant to reinfection with the intestinal coccidian parasite *Eimeria nieschulzi* or the nematode *Nippostrongylus brasiliensis*. Parasite Immunology, 1, 125-132.

ROSE, M.E., PEPPARD, J.V. and HOBBS, S.M. (1984). Coccidiosis: characterisation of antibody responses to infection with *Eimeria nieschulzi*. Parasite Immunology, 6, 1-12.

ROSE, M.E., WAKELIN, D., JOYSEY, H.S. and HESKETH, P. (1988). Immunity to coccidiosis: adoptive transfer in NIH mice challenged with *Eimeria vermiformis*. Parasite Immunology, 10, 59-69.

RYLEY, J.F. (1973). Cytochemistry, physiology and biochemistry. In: The Coccidia: *Eimeria, Isospora, Toxoplasma* and Related Genera (ed. D.M. Hammond and P.L. Long) pp 162-165. University Park Press, Baltimore and Butterworth and Co. (Publishers) Ltd., London.

SAIF, L.J. (1987). Development of nasal, faecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. Veterinary Immunology and Immunopathology, 17, 425439.

SANFORD, S.E. (1987). Enteric cryptosporidial infection in pigs: 184 cases (1981-1985). Journal of the American Veterinary Medical Association, 190, 695-698.

SANFORD, S.E., and JOSEPHSON, G.K.A. (1982). Bovine cryptosporidiosis: clinical and pathological findings in forty-two scouring neonatal calves. Canadian Veterinary Journal, 23, 343-347.

SATTAR, S.A., RAPHAEL, R.A. and SPRINGTHORPE, V.S. (1983). Rotaviruses and chemical disinfectants. In: Proceedings, Fourth International Symposium on Neonatal Diarrhoea, VIDO, Saskatoon, University of Saskatchewan, pp 90-99.

SAXON, A. (1987). Oral administration of bovine colostrum anti-cryptosporidia antibody fails to alter the course of human cryptosporidiosis. Journal of Parasitology, 73, 413-415.

SCHOLTYSECK, E. (1973). Ultrastructure. In: The Coccidia: *Eimeria, Isospora, Toxoplasma* and Related Genera (ed. D.M. Hammond and P.L. Long), pp 81-144. University Park Press, Baltimore and Butterworths and Co. (Publishers) Ltd., London.

SHAPIRO, A.L., VINUELA, E. and MAIZEL, J. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemical and Biophysical Research Communications, 28, 815-820.

SHELDRAKE, R.F., HUSBAND, A.J., WATSON, D.L., BARGER, I.A. and BORAY, J.C. (1988). Transport of serum IgA into bile of sheep infected with *Fasciola hepatica*. Veterinary Immunology and Immunopathology, 18, 29-39.

SHELDRAKE, R.F., HUSBAND, A.J., WATSON, D.L. and CRIPPS, A.W. (1984). Selective transport of serum-derived IgA into mucosal secretions. Journal of Immunology, 132, 363-368.

SHERWOOD, D., ANGUS, K.W., SNODGRASS, D.R. and TZIPORI, S. (1982). Experimental cryptosporidiosis in laboratory mice. Infection and Immunity 38, 471-475.

SIEGEL, S. (1956). In: Nonparametric Statistics for the Behavioural Sciences. pp 95-156. McGraw-Hill Book Company, Inc.

SMITH, W.D., WELLS, P.W., BURRELLS, C. and DAWSON, A.MCL. (1976). Maternal immunoglobulins and parainfluenza 3 virus inhibitors in the nasal and lachrymal secretions and serum of newborn lambs. Clinical and Experimental Immunology, 23, 544-553.

SNEDECOR, G.W. and COCHRAN, W.G. (1967). In: Statistical Methods. 6th edn, p 59. Ames, Iowa: The Iowa State University Press.

SNIDER, D.P. and UNDERDOWN, B.J. (1986). Quantitative and temporal analyses of murine antibody response in serum and gut secretions to infection with *Giardia muris*. Infection and Immunity, 52, 271-278.

SNODGRASS, D.R., ANGUS, K.W., GRAY, E.W., KEIR, W.A. and CLERIHEW, L.W. (1980). Cryptosporidia associated with rotavirus and an *Escherichia coli* in an outbreak of calf scour. Veterinary Record, 106, 458-459.

SNODGRASS, D.R., ANGUS, K.W. and GRAY, E.W. (1984). Experimental cryptosporidiosis in germ free lambs. Journal of Comparative Pathology, 94, 141-152.

SNODGRASS, D.R. and SHERWOOD, D. (1983). Aetiology of diarrhoea in young calves. In: Proceedings, 4th International Symposium on Neonatal Diarrhoea, VIDO, Saskatoon, University of Saskatchewan, pp 162-170.

SNYDER, S.P., ENGLAND, J.J. and McCHESNEY,A.E. (1978). Cryptosporidiosis in immunodeficient Arabian foals. Veterinary Pathology, 15, 12-17.

SOAVE, R. and JOHNSON, Jr., W.D. (1988). *Cryptosporidium* and *Isospora belli* infections. Journal of Infectious Diseases, 157, 225-229.

TAKEUCHI, A. (1971). Penetration of the intestinal epithelium by various microorganisms. Current Topics in Pathology, 54, 1-27.

TYZZER, E.E. (1907). A sporozoan found in the peptic glands of the common mouse. Proceedings of the Society for Experimental Biology and Medicine, 5, 12-13.

TYZZER, E.E. (1910). An extracellular coccidium, *Cryptosporidium muris* (gen. et sp. nov.), of the gastric glands of the common mouse. Journal of Medical Research, 23, 487-509.

TYZZER, E.E. (1912). *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. Archiv für Protistenkunde, 26, 394-413.

TZIPORI, S. (1983). Cryptosporidiosis in animals and humans. Microbiological Reviews, 47, 84-96.

TZIPORI, S. (1985). The relative importance of enteric pathogens affecting neonates of domestic animals. In: Advances in Veterinary Science and Comparative Medicine (ed. C.F. Cornelious and C.F. Simpson), 29, 103-206. New York: Academic Press. Cited from: TZIPORI, S. (1985). *Cryptosporidium*: notes on epidemiology and pathogenesis. Parasitology Today, 1, 159-165.

TZIPORI, S. (1988). Cryptosporidiosis in perspective. In: Advances in Parasitology (ed. J.R. Baker and R. Muller), 27, 63-129. Academic Press (Harcourt Brace, Jovanovich, Publishers).

TZIPORI, S. and CAMPBELL, I. (1981). Prevalence of *Cryptosporidium* antibodies in 10 animal species. Journal of Clinical Microbiology, 14, 455-456.

TZIPORI, S., ANGUS, K.W., CAMPBELL, I. and GRAY, E.W. (1980a). *Cryptosporidium*: evidence for a single-species genus. Infection and Immunity, 30, 884-886.

TZIPORI, S., CAMPBELL, I., SHERWOOD, D., SNODGRASS, D.R. and WHITELAW, A. (1980b). An outbreak of calf diarrhoea attributed to cryptosporidial infection. Veterinary Record, 107, 579-580.

TZIPORI, S., ANGUS, K.W., CAMPBELL, I. and CLERIHEW, L.W. (1981a). Diarrhoea due to *Cryptosporidium* infection in artificially reared lambs. Journal of Clinical Microbiology, 14, 100-105.

TZIPORI, S., ANGUS, K.W., CAMPBELL, I. and SHERWOOD, D. (1981b). Diarrhoea in young red deer associated with infection with *Cryptosporidium* . Journal of Infectious Diseases, 144, 170-175.

TZIPORI, S., ANGUS, K.W., GRAY, E.W., CAMPBELL, I. and ALLEN, F. (1981c). Diarrhoea in lambs experimentally infected with *Cryptosporidium* isolated from calves. American Journal of Veterinary Research, 42, 1400-1404.

TZIPORI, S., McCARTNEY, E., LAWSON, G.H.K., ROWLAND, A.C. and CAMPBELL, I. (1981d). Experimental infection of piglets with *Cryptosporidium*. Research in Veterinary Science, 31, 358-368.

TZIPORI, S., SHERWOOD, D., ANGUS, K.W., CAMPBELL, I. and GORDON, M. (1981e). Diarrhoea in lambs: experimental infections with enterotoxigenic *Escherichia coli*, rotavirus and *Cryptosporidium*. Infection and Immunity, 33, 401-406.

TZIPORI, S., ANGUS, K.W., CAMPBELL,I. and GRAY, E.W. (1982a). Experimental infection of lambs with *Cryptosporidium* isolated from a human patient with diarrhoea. Gut, 23, 71-74.

TZIPORI, S., LARSEN, J., SMITH, M. and LUEFL, R. (1982b). Diarrhoea in goat kids attributed to *Cryptosporidium* infection. Veterinary Record, 111, 35-36.

TZIPORI, S., ROBERTSON, D. and CHAPMAN, C. (1986). Remission of cryptosporidiosis in an immunodeficient child with hyperimmune bovine colostrum. British Medical Journal, 293, 1276-1277.

TZIPORI, S., SMITH, M., HAPLIN, C., ANGUS, K.W., SHERWOOD, D. and CAMPBELL, I. (1983). Experimental cryptosporidiosis in calves: clinical manifestations and pathological findings. Veterinary Record, 112, 116-120.

TZIPORI, S., SMITH, M., MAKIN, T. and McCAUGHAN, C. (1978). Enteric coronavirus-like particles in sheep. Australian Veterinary Journal, 54, 320-321.

UNGAR, B.L.P. and NASH, T.E. (1986). Quantification of specific antibody response to *Cryptosporidium* antigens by laser densitometry. Infection and Immunity, 53, 124-128.

UNGAR, B.L.P., SOAVE, R., FAYER, R. and NASH, T.E. (1986). Enzyme immunoassay detection of immunoglobulin M and G antibodies to *Cryptosporidium* in immunocompetent and immunocompromised persons. Journal of Infectious Diseases, 153, 570-578.

VAN DER HEIJDEN, P.J., BIANCHI, A.T.J., STOK, W. and BOKHOUT, B.A. (1988). Background (spontaneous) immunoglobulin production in the murine small intestine as a function of age. Immunology, 65, 243-248.

VAN REES, E.P., DIJKSTRA, C.D., VAN DER ENDE, M.B., JANSE, E.M. and SMINIA, T. (1988). The ontogenetic development of macrophage subpopulations and Ia-positive non-lymphoid cells in gut-associated lymphoid tissue of the rat. Immunology, 63, 79-85.

VETTERLING, J.M., JERVIS, H.R., MERRILL, T.G. and SPRINZ, H. (1971a). *Cryptosporidium wrairi* (sp. n.) from the guinea pig (*Cavia porcellus*) with an emendation of the genus. Journal of Protozoology, 18, 243-247.

VETTERLING, J.M., TAKEUCHI, A. and MADDEN, P.A. (1971b). Ultrastructure of *Cryptosporidium wrairi* from the guinea pig. Journal of Protozoology, 18, 248-260.

WAKELIN, D. (1984). Analysis of Immunity. In: Immunity to Parasites. How Animals Control Parasite Infections. p 141. London: Edward Arnold.

WALDMAN, E., TZIPORI, S. and FORSYTH, J.R.L. (1986). Separation of *Cryptosporidium* species oocysts from faeces by using a Percoll discontinuous density gradient. Journal of Clinical Microbiology, 23, 199-200.

WATSON, D.L. and LASCELLES, A.K. (1973). Mechanisms of transfer of immunoglobulins into mammary secretions of ewes. Australian Journal of Experimental Biology and Medical Science, 51, 247-254.

WEISER, M.M. (1973). Intestinal epithelial cell surface membrane glycoprotein synthesis I. An indicator of cellular differentiation. Journal of Biological Chemistry, 248, 2536-2541.

WILDERS, M.M., SMINIA, T., and JANSE, E.M. (1983). Ontogony of non-lymphoid and lymphoid cells in the rat gut with special reference to large mononuclear Ia-positive dendritic cells. Immunology, 50, 303-314.

WILLSON, P.J. and ACRES, S.D. (1982). A comparison of dichromate solution flotation and faecal smears for diagnosis of cryptosporidiosis in calves. Canadian Veterinary Journal, 23, 240-246.

WILSON, M.B. and NAKANE, P.K. (1978). Recent developments in the periodate method of conjugating horseradish peroxidase to antibodies. In: Immunofluorescence and related staining techniques (ed. W. Knapp, K. Holubar and G. Wick), pp 215-224. Elsevier/North -Holland Biomedical Press.

WOODMANSEE, D.B. (1986). An *in vitro* study of sporulation in *Cryptosporidium* species. Journal of Parasitology, 72, 348-349.

PUBLICATIONS

The following are publications arising from the work described in this thesis:

HILL, B.D., BLEWETT, D.A., DAWSON, A.M. and WRIGHT, S. (1988). Analysis of the kinetics, isotype and specificity of serum and coproantibody in lambs infected with *Cryptosporidium*. Research in Veterinary Science (in press).

HILL, B.D. (1988). Immune responses in *Cryptosporidium* Infections. In: Cryptosporidiosis, Proceedings of the First International Workshop (ed. K.W. Angus and D.A. Blewett), pp97-105. Edinburgh, Dupli-quick printers.

APPENDIX 1. The origin, passage number and identification of *Cryptosporidium* isolates used during this study.

Isolate identification and origin	Laboratory passage number	Experimental use
B[1]: faeces from a 14 day old calf; Penrith, Cumbria; June, 1983.	15(B/'86/10)[2]	Experiment 3.1
E[1]: faeces from a 10 day old foal; Stirling, Stirlingshire; May, 1986.	2(E/'86/9)	Experiment 3.1
C[1]: faeces from a 10 day old red deer calf; Glensaugh, Grampian; June, 1986.	1(C/'86/7)	Experiment 3.3
	2(C/'86/10)	" 3.1
	3(C/'87/1)	" 3.2
	4(C/'87/4)	" 3.5 (colostrum deprived lambs)
	5(C/'87/8)	Experiment 3.4
	6(C/'87/12)	" 3.5 (gnotobiotic lambs)
	7(C/'88/3)	Chapter 6: *In vivo* and *in vitro* sporozoite studies
	8(C/'88/6)	

1. The three isolates of *Cryptosporidium* used in this study were of bovine(B), equine(E) and cervine(C) origin, having been isolated from the faeces of neonates of these species in the United Kingdom.

2. The laboratory passage number since isolation from the wild is followed, in brackets, by the isolate identification/ year/ and month of passage. Only passages from which oocysts were used in the present study are shown.